UNIVERSITY OF ALBERTA

PHYSICAL AND FUNCTIONAL INTERACTIONS OF THE HEMATOPOIETIC CELL-SPECIFIC PROTEIN-TYROSINE PHOSPHATASE CD45

BY

CHRISTOPHER WILLIAM ARENDT

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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June 3, 1998

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **PHYSICAL AND FUNCTIONAL INTERACTIONS OF THE HEMATOPOIETIC CELL-SPECIFIC PROTEIN-TYROSINE PHOSPHATASE CD45** submitted by **CHRISTOPHER WILLIAM ARENDT** in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in IMMUNOLOGY.

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ABSTRACT

CD45 is a transmembrane protein-tyrosine phosphatase expressed on all nucleated hemopoietic cells. CD45 functions as a critical mediator of T-cell activation responses by regulating Src family protein-tyrosine kinases such as $p56^{lck}$. It is not known how CD45 activity is itself regulated or whether CD45 performs additional functions within a range of hematopoietic cell types. This study seeks to apply both immunological and biochemical methodologies in investigating potential novel functions of CD45.

Although CD45 possesses a receptor-like structure and different isoforms of the molecule displaying heterogeneity in the extracellular domain are expressed in a developmentally restricted manner, a specifically interacting ligand has not been identified. To characterize the biological effects of CD45 ligation, T-cell responses to immobilized antibodies to CD45 have been examined. Engagement of CD45 in this manner induces dramatic changes in cell morphology that are dependent upon both actin polymerization and herbimycin A-sensitive kinase activity. In a coordinated manner, tyrosine phosphorylation of a variety of $p56^{lck}$-associated proteins is observed. Intriguingly, both the morphologic changes and phosphorylation events require the expression of $p56^{lck}$. Together, the data suggest that CD45 is capable of initiating a cytoskeletal-remodeling cascade that may be relevant to processes such as cellular migration or conjugate formation.

To fully understand the biological functions of CD45, it is necessary to identify proteins with which it interacts in various cellular contexts. Such proteins may regulate the enzymatic activity of CD45 or perform unexpected functions that provide new

insights into the biology of this phosphatase. Two proteins that interact with CD45 in a stable and specific manner have been identified. Purification of these proteins and subsequent cDNA cloning have revealed that both are multiple isoform proteins that function as the α- and β-subunits of glucosidase II, an exoglucohydrolase of the endoplasmic reticulum. Interestingly, the ability of glucosidase II to stably interact with CD45 exhibits cell-type specificity in that it generally does not occur in more mature cells. Physical aspects underlying the intermolecular association of these proteins have been investigated. The cell-specific association of glucosidase II with CD45 may impinge on the spectrum of oligosaccharides presented by CD45 at the cell surface.

This thesis is dedicated to the memory of my grandfather

William Joseph McVea

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ABBREVIATIONS

- Ab — Antibody
- Ag — Antigen
- AGE — Advanced glycation endproduct
- APC — Antigen presenting cell
- ATCC — American Type Culture Collection (Bethesda, MD)

- BSA — Bovine serum albumin

- $[Ca^{2+}]_i$ — Intracellular free calcium concentration
- cDNA — Complementary DNA
- CNBr — Cyanogen bromide
- CTL — Cytotoxic T lymphocyte
- cyt D — Cytochalasin D
- cyt E — Cytochalasin E

- DAG — Diacylglycerol
- DCS — Defined calf serum
- DNA — Deoxyribonucleic acid
- DMEM — Dulbecco's modified Eagles' medium
- dNTP — Dinucleotide triphosphate
- DOC — Deoxycholate

- ECL — Enhanced chemiluminescence
- EGFR — Epidermal growth factor receptor
- Endo F — Endo-β-*N*-acetylglucosaminidase F (includes *N*-glycanase F)
- Endo H — Endo-β-*N*-acetylglucosaminidase H
- ER — Endoplasmic reticulum

- FACS — Fluorescence-activated cell sorter
- FCS — Fetal calf serum
- FITC — Fluorescein isothiocyanate

- GII_{α} — α-subunit of glucosidase II
- GII_{β} — β-subunit of glucosidase II
- Glc — Glucose
- GlcNAc — *N*-acetylglucosamine
- GST — Glutathione S-transferase

- h — Hour(s)
- HDEL — His-Asp-Glu-Leu
- herb A — Herbimycin A
- HRP — Horseradish peroxidase

- IL — Interleukin
- IP_3 — Inositol 1,4,5-trisphosphate
- IPTG — Isopropyl β-D-thiogalactopyranoside
- ITAM — Immunoreceptor tyrosine-based activation motif

- kb — Kilobase pair(s)

- LAG — Lysosomal α-glucosidase
- LFA-1 — CD11a/CD18

- mAb — Monoclonal antibody
- 2-ME — 2-Mercaptoethanol
- Man — Mannose
- min — Minute(s)
- MHC — Major histocompatibility complex
- mRNA — Messenger RNA

- NP-40 Nonidet P-40

- OD Optical density
- ORF Open reading frame

- p80 CD45-associated protein of 80 kDa
- p116 CD45-associated protein of 116 kDa
- PAGE Polyacrylamide gel electrophoresis
- PBS Phosphate-buffered saline (pH 7.4)
- PCR Polymerase chain reaction
- PKC Protein kinase C
- PLC Phospholipase C
- PTK Protein-tyrosine kinase
- PTP Protein-tyrosine phosphatase

- RACE Rapid amplification of cDNA ends
- RNA Ribonucleic acid
- rpm Rotations per minute
- RT Reverse transcription

- s Second(s)
- SDS Sodium dodecyl sulfate

- TCR T-cell antigen receptor
- TPA 12-0-Tetradecanoylphorbol-13-acetate

- U Unit(s)
- UDP-Glc Uridine diphosphate glucose
- UGGT UDP-glucose:glycoprotein glucosyltransferase

CHAPTER I

GENERAL INTRODUCTION

A. The Specific Immune Response

One of the most fascinating features of the mammalian immune system is the remarkable acuity with which infectious pathogens are recognized and eliminated. In order to safeguard against disease and death, the immune system must be capable of neutralizing harmful bacterial toxins while ignoring endogenous hormones, killing cells harboring virus while leaving undamaged casein producing mammary cells that become activated during pregnancy. An exquisitely complex but elegant system of antigen (Ag) presentation has evolved to facilitate immune surveillance (Germain, 1994). Macrophages and dendritic cells, which function as Ag presenting cells (APCs), mediate the endocytosis of exogenous proteins that are cleaved by endosomal peptidases into small peptide fragments capable of being presented at the cell surface in complex with major histocompatibility complex (MHC) class II proteins. A specific class of T cells termed $CD4^+$ cells because they express the CD4 surface glycoprotein that interacts with class II molecules, continuously survey the diverse spectrum of class II-associated peptides presented by APCs. Counterbalancing this system is a ubiquitous Ag processing pathway whereby peptides derived from intracellular proteins are presented by MHC class I molecules that are surveyed by $CD8^+$ T lymphocytes. Together, these two

complementary Ag processing pathways can be conceptualized as providing an overview of all the proteins present within an organism at a given moment in time.

The structural basis for recognition of Ag by T lymphocytes has been previously elucidated (Fields and Mariuzza, 1996). All T cells express a unique heterodimeric T-cell receptor (TCR) capable of recognizing both MHC proteins and the peptides contained therein. The inherent diversity of the antigenic universe is mirrored in the diversity of the TCR repertoire, with TCR specificity being determined independently in each cell through random gene rearrangement and mutational events. Through a complex series of developmental events occurring in the thymus, only T cells expressing TCRs capable of recognizing self-MHC molecules, but not peptides derived from self proteins, are permitted to survive (Nossal, 1994). In this way, the TCR repertoire is restricted to self-MHC proteins and, by default, non-self antigens. Moreover, through mechanisms that are not completely understood, thymic emigrants possessing class I-directed TCRs express CD8, while T cells capable of recognizing class II proteins express CD4 (von Boehmer, 1994).

During a primary immune response, $CD4^+$ T cells capable of recognizing pathogen-derived peptides (e.g. derived from circulating virus proteins) become activated upon encountering these peptides in association with class II molecules on APCs. The $CD4^+$ subset is also dubbed "helper" T cells because of the ability of these cells to secrete soluble cytokines that function in a paracrine manner to stimulate the proliferation of other lymphocytes, namely $CD8^+$ T cells and B lymphocytes (Mosmann and Sad, 1996). $CD8^+$ T cells, when activated by the appropriate complex of Ag/class I (e.g. on the surface of a virally infected cell) mature into cytotoxic T lymphocytes (CTLs) capable of

mediating the destruction of infected cells by a variety of mechanisms (Atkinson and Bleackley, 1995). B lymphocytes, in contrast, possess Ab surface receptors with specificity and diversity paralleling that of the TCR, but that function to bind directly to unprocessed soluble proteins (e.g. virus coat proteins). B cells, when activated in the presence of Ag and helper factors from $CD4^+$ T cells, differentiate into plasma cells that secrete high levels of soluble Ab (Parker, 1993). Soluble Ab mediates a variety of protective functions, including neutralization of toxins, activation of complement-mediated lysis, and triggering of scavenger cells to eliminate Ab-coated pathogens. Thus, the ability of the immune system to respond rapidly to infection depends on cooperation between multiple cell types whose activation is governed by an ingenious system of Ag processing and presentation. Because lymphocytes exhibit easily measurable functional responses upon activation, and because these cells can be cloned and maintained *in vitro*, they offer an ideal system for the examination of molecular events regulating these outcomes.

B. Principles of Lymphocyte Activation

Studies of naïve T cells have revealed that two types of signals are required for full cellular activation (Matzinger, 1994, and Langman and Cohn, 1996). The T cell receives "signal 1" when its TCR recognizes Ag/MHC. "Signal 2" is provided to the cell in the form of a costimulatory signal that may take on a variety of forms (Liu et al., 1992; Zuckerman et al., 1995; Taub et al., 1996), one example being activation of a signaling cascade by ligation of the T-cell surface protein CD28 by B7-1/B7-2 expressed on the

APC (Rudd, 1996). Only upon simultaneous delivery of these two signals are nuclear activation events triggered that allow a naïve T cell to proliferate and differentiate into an activated cell capable of expressing its effector functions (Su et al., 1994; Bachmann et al., 1997). If signal 1 is received in the absence of signal 2, the cell may enter a state of prolonged functional ignorance, known as anergy (Schwartz, 1996), which is characterized by irreversible blockade of signal transduction pathways required for synthesis of the autocrine growth factor IL-2 (Li et al., 1996a). This pathway has been hypothesized to serve a protective function by restricting inductive events to professional APCs capable of delivering signal 2. In this way, any self-reactive T lymphocyte that manages to escape the thymus will be rendered anergic due to the high probability of encountering signal 1 (i.e. self peptide) on a non-professional APC incapable of providing signal 2. Once a naïve T cell has been provided with both signal 1 and 2 together, it differentiates into a cell capable of mediating effector functions in response to signal 1 alone.

Recent studies have built upon the two signal model of lymphocyte activation by considering additional variables, such as the strength of the TCR-Ag/MHC interaction (binding affinity), TCR and Ag/MHC expression levels (which influence binding avidity), duration of stimulation, contribution of adhesion molecules, and costimulation conditions (Kim et al., 1996; Kundig et al., 1996; Schodin et al., 1996; Viola and Lanzavecchia, 1996; Wulfing et al., 1997; Iezzi et al., 1998). A common theme to emerge from this work is that T-cell activation can be viewed in terms of discrete "signaling thresholds" which must be overcome in order for activation events to be achieved. In addition, different functional responses such as cell proliferation, cytokine

secretion, and release of cytolytic granules appear to be governed by distinct activation potentials (Valitutti et al., 1996). Intense efforts have focused on elucidating the mechanisms by which extracellular stimuli are translated into intracellular signals that are integrated at the level of the nucleus to determine the outcomes of cellular activation.

C. Early Signal Transduction Events in T Lymphocytes

Intracellular signal transduction events have been intensely examined in T cells stimulated with specific antigen, or antibodies (Abs) to the TCR complex (Chan et al., 1994). The TCR complex consists of several physically associated proteins, including the α and β chains of the TCR (which are covalently linked as heterodimers), and various covalently and noncovalently associated members of the CD3 family of proteins, including ζ, η, δ, ε and μ. The earliest detectable event in lymphocytes that occurs within seconds of Ag stimulation is PTK activation. The activation of one or more intracellular PTKs leads rapidly to phosphorylation and activation of PLCγ, which elicits the production of DAG and IP_3 lipid mediators. DAG remains associated with the cell membrane, recruiting and activating PKC-θ (Monks et al., 1997), while IP_3 stimulates a transient rise in $[Ca^{2+}]_i$ by depleting intracellular Ca^{2+} stores (Premack and Gardner, 1994). Depletion of intracellular Ca^{2+} stores triggers a sustained increase in $[Ca^{2+}]_i$ by activating Ca^{2+} influx across the plasma membrane (Fanger et al., 1995). Both the accumulation of $[Ca^{2+}]_i$ and PKC activation are necessary to activate signal transduction cascades that culminate, in T cells, in the upregulation of IL-2 transcription (Crabtree and Clipstone, 1994). The production of this cytokine has an autocrine regulatory effect,

stimulating cell-cycle progression in a Ca^{2+} and PKC-independent manner. Thus, various classes of molecules including proteins, ions, and lipids collaborate in the early signal transduction events associated with T-cell activation.

The rapid triggering of reversible phosphorylation events on tyrosine residues during T-cell activation is of particular interest, since these events are relatively rare (<5%) in comparison to those occurring on serine or threonine (Perlmutter et al., 1993). Despite their rarity, tyrosine phosphorylation events have been shown to be strongly associated with the regulation of cellular proliferation and differentiation pathways. The rapid kinetics with which tyrosine phosphorylation of cellular proteins is upregulated upon activation was at first surprising given that none of the members of the TCR complex possess integral enzymatic activity. This discrepancy was resolved upon the discovery that the Src family PTK $p56^{lck}$ is physically associated with the cytoplasmic tails of the CD8 and CD4 coreceptors (Barber et al., 1989; Shaw et al., 1989). The ability of CD8/CD4 to ligate class I/class II proteins at sites of TCR engagement thus provides a possible mechanism by which intracellular PTK activity is recruited to the TCR complex.

D. CD45: A Transmembrane Protein-tyrosine Phosphatase Functioning in Lymphocyte Activation

The finding that PTK activity is rapidly triggered upon lymphocyte activation fits well with the positive correlation between tyrosine phosphorylation and activation of growth responses. More surprising was the revelation that PTK activation is uncoupled from Ag receptor stimulation in the absence of a cellular PTP known as CD45

(Trowbridge and Thomas, 1994). CD45 is a high molecular weight (~180 to ~220 kDa) transmembrane PTP expressed on all cells of hematopoietic lineage except erythrocytes (Fig. 1-1). CD45 is an abundant protein, expressed at approximately 100,000-200,000 molecules per cell and accounting for roughly 10% of the total membrane protein mass. To date, eight isoforms of CD45 have been identified that arise from a single by differential usage of three small exon cassettes (exons 4-6, termed A-C for simplicity) encoding an amino-terminal region of the ectodomain. Of particular experimental utility in identifying particular subtypes of cells, these isoforms are expressed in a cell type-specific and differentiation stage-specific manner (Tung et al., 1984; Ewald and Refling, 1985; Lefrancois et al., 1986; Lefrancois and Goodman, 1987; Saga et al., 1987; Pulido et al., 1988; Pulido et al, 1989; Saga et al., 1990; Hamann et al., 1997), although the relevance of this highly coordinated genetic regulation is uncertain. Splenic B cells, for example, are known to express high molecular weight CD45 isoforms, while low molecular weight isoforms are expressed by immature thymocytes and memory T cells. Further heterogeneity is contributed by glycosylation, which is influenced both by the variable exon repertoire and cell-specific controls (Lefrancois and Bevan, 1985; Lefrancois, 1987; Pulido and Sanchez-Madrid, 1989). The region encoded by exons A-C is rich in Ser and Thr residues and is known to be highly *O*-glycosylated (Pulido and Sanchez-Madrid, 1990), thus conferring significant negative charge on the amino-terminal domain. Up to 18 sites of *N*-linked glycosylation are also present within ectodomain of mouse CD45, though only 3 potential sites are contained within exons A-C (Johnson et al., 1989a). Apparently, many of these *N*-linked sites are indeed utilized, since Endo F-sensitive carbohydrate accounts for approximately one quarter of the

molecular mass of CD45 (Uemura et al., 1996). A final notable feature of the CD45 extracellular domain is that the more membrane proximal region contains two Cys-rich clusters for a total of 17-18 Cys residues (Johnson et al., 1989a). No evidence has been obtained that these sites form intermolecular linkages, and CD45 is believed to exist as a monomer in resting cells (Trowbridge and Thomas, 1994).

All isoforms of CD45 share a large, highly conserved cytoplasmic domain containing two subdomains bearing significant homology to placental PTP 1B (Charbonneau et al., 1988). The more membrane proximal of these, termed domain I, has been shown to possess intrinsic PTP activity and is capable of dephosphorylating a wide variety of artificial substrates (Tonks et al., 1988; Streuli et al., 1990). The activity of this domain is dependent upon the integrity of a catalytic Cys residue (Streuli et al., 1989). Domain II, the second subdomain, possesses a more divergent catalytic consensus sequence and has not been convincingly shown to possess catalytic function (Streuli et al., 1989; Johnson et al., 1992). Site-directed mutagenesis of domain II to reconstitute a catalytic consensus sequence identical to that present in domain I failed to restore the enzymatic function of domain II, indicating that other undefined defects constrain the activity of this domain (Johnson et al., 1992). Interestingly, point mutations or deletions in domain II have been found to affect the catalytic activity of domain I, raising the possibility that domain II may function to regulate the enzymatic activity or substrate specificity of the first subdomain (Streuli et al., 1990; Johnson et al., 1992; Iida et al., 1994). Reflecting its robust expression, CD45 has been found to account for more than 90% of the total membrane-associated PTP activity in a T-cell line (Mustelin et al., 1989).

Considerable excitement was stirred by a number of studies demonstrating a critical role for CD45 in lymphocyte activation. The first observation by Pingel and Thomas (1989) was that a $CD4^+$ T cell clone lacking surface expression of CD45 did not proliferate in response to TCR triggering, although it remained capable of responding to the growth factor IL-2. Examination of intracellular second messenger production in a CD45-negative T-line revealed that TCR-stimulated phosphatidyl inositol turnover was ablated (Koretzky et al., 1990). A CD45-deficient $CD8^+$ CTL clone was also obtained that exhibited greatly impaired cytolytic function, proliferation, and cytokine secretion upon TCR triggering (Weaver et al., 1991). In each of these studies revertants of the mutant cells were isolated that reexpressed CD45 and, in each case, the specific defects were corrected. Finally, it was demonstrated that Ag-triggered $[Ca^{2+}]_i$ mobilization is impaired in CD45-deficient B cells (Justement et al., 1991) and that CD45-negative T cells exhibit reduced, delayed, and irregular Ca^{2+} fluxes in response to TCR triggering due to disregulated mobilization of intracellular Ca^{2+} stores (Volarevic et al., 1992).

Although the above studies dramatically and convincingly revealed an important role for CD45 in regulating responses to Ag, it was uncertain to what extent the PTP activity of CD45 mediated these functions. To address this issue, several groups reconstituted CD45-negative cells with chimeric molecules containing the cytoplasmic domain of CD45 joined to the myristylated amino terminus of $p60^{c\text{-}src}$ (Volarevic et al., 1993), to the transmembrane and extracellular domains of class I (Hovis et al., 1993), or to the transmembrane region and external segment of the epidermal growth factor receptor (EGFR) (Desai et al., 1993). In each instance, TCR-mediated signals were restored to near wild type levels, although both qualitative (Volarevic et al., 1993) and

quantitative (Hovis et al., 1993; Desai et al., 1993) differences in certain signal transduction events were apparent. When the PTP activity of the CD45/EGFR chimera was ablated by mutagenesis, signal transduction could not be reconstituted, indicating that the enzymatic function of CD45 is necessary for lymphocyte activation (Desai et al., 1994). Remarkably, cell surface expression of a yeast single domain nonreceptor PTP in a variant of Jurkat deficient in CD45 permitted the restoration of TCR signal transduction to near normal levels, a phenomenon dependent upon the enzymatic activity of this chimera (Motto et al., 1994). Collectively, these studies indicate that membrane-associated PTP activity is essential for Ab-triggered T-cell activation and that the extracellular segment of CD45 is dispensable in this context. However, this work by no means excludes the possibility that the external domain of CD45 exerts physiologically relevant controls on responses *in vivo*.

Together, the early studies of CD45 employing mutant cell lines suggested that it functions as an on/off switch in regulating immune responsiveness. More recently, this view has been challenged by gene ablation experiments in mice. Kishihara et al. (1993) generated mice bearing a homozygous deletion in exon 6, the alternatively spliced exon C. This defect ablated expression of exon 6-specific isoforms of CD45, such as those expressed on B cells, and also decreased the efficiency of expression of exon 6-negative CD45 isoforms on other hematopoietic subsets. Interestingly, B cell development appeared to be grossly normal in these mice, and peripheral B cells were capable of mobilizing Ca^{2+} from internal stores, although extracellular Ca^{2+} influx was abrogated and the cells were unresponsive to IgM crosslinking (Benatar et al., 1996). Targeting of exon 9 of CD45 permitted generation of mice truly negative for CD45 expression (Byth

et al., 1996). Major defects were observed in positive and negative thymocyte selection, although some T cells were capable of populating the periphery. The fact that T cell maturation was not completely ablated indicates that CD45 is not simply an on/off switch but is more likely to regulate the thresholds of TCR signaling pathways. Consistent with this concept, CD45-null thymocytes were found to be capable of mediating proximal signals such as Ca^{2+} mobilization and inositol phosphate turnover (Stone et al., 1997). While it is always necessary to interpret gene ablation experiments with caution, the results support a model wherein CD45 functions to potentiate activation by decreasing signaling thresholds.

E. Regulation of Src family Protein Tyrosine Kinases by CD45

That a PTP should be capable of exerting a positive regulatory influence on cellular activation was counterintuitive to the notion that tyrosine phosphorylation is required for cell differentiation and proliferation. A major step towards resolving this paradox was taken by Ostergaard et al. (1989), who demonstrated that $p56^{lck}$ is hyperphosphorylated on carboxyl-terminal Tyr-505 in three independently derived CD45-negative cell lines. Similarly, Tyr-531 of $p56^{fyn}$, another Src family kinase expressed in T cells, was found to be hyperphosphorylated in CD45-negative lymphoma cells, although the effect was not as dramatic as that observed with $p56^{lck}$ (Hurley et al., 1993). Based on a variety of evidence, CD45 was hypothesized to act upon these kinases directly (Ostergaard and Trowbridge, 1990; Koretzky et al., 1993; Ng et al., 1996). A major question remaining untested was the relevance of CD45-mediated

dephosphorylation to the enzymatic activity of these Src family members. Given the correlation between lack of CD45 expression, hyperphosphorylation of Src family PTKs, and inhibition of PTK activity associated with cellular activation, it seemed reasonable to surmise that the carboxyl-terminal Tyr on $p56^{lck}$ and $p59^{fyn}$ recognized by CD45 constitutes a negative regulatory site (Fig. 1-2). By analogy to $p60^{c\text{-}src}$ (Cantley et al., 1991; Koch et al, 1991), a paradigm was invoked (and rapidly accepted as dogma) in which a negatively regulatory Tyr phosphate group is capable of interacting in an intramolecular fashion with the SH2 present in these Src family PTKs, resulting in occlusion of the catalytic kinase domain (Mustelin and Burn, 1993). According to this scheme, CD45 is required to release $p56^{lck}$ and $p59^{fyn}$ from autoinhibition, allowing these PTKs to become activated during the initiation of immune responses.

While attractive in its simplicity, experiments aimed at testing the veracity of this model in a variety of cell systems have yielded conflicting results. Consistent with the model, Cahir McFarland et al. (1993) observed that both $p56^{lck}$ and $p59^{fyn}$ are hyperphosphorylated and hypoactive in a CD45-deficient $CD8^+$ T cell clone. This finding was corroborated in CD45-null thymocytes from the exon 9 -/- mouse (Stone et al., 1997). In contrast, Ostergaard and Trowbridge (1990) reported that Ab coclustering of CD45 and CD4 results in dephosphorylation of CD4-associated $p56^{lck}$, coincident with downregulation of its PTK activity. Burns et al. (1994) observed that in CD45-negative T lymphoma cells, $p56^{lck}$ and $p59^{fyn}$ are constitutively hyperphosphorylated and display augmented kinase activity. Interestingly, this group was able to demonstrate that Tyr-505 was indeed hyperphosphorylated in these cells, thus calling into question the ability of this phosphotyrosine residue to mediate a dominant inhibitory effect. A similar

examination of CD45-negative macrophages yielded identical results, demonstrating that this phenomenon is not T-cell specific (Roach et al., 1997).

These discrepant findings suggest a number of possible resolutions. One complication to these studies is that Src family PTKs possess reversible lipid modifications that permit localization to various subcellular environments, including the plasma membrane, the cytoplasm and, possibly, intracellular membranes (Resh, 1994; Yurchak and Sefton, 1995). Indeed, evidence has been obtained that cell-surface pools of CD4-associated $p56^{lck}$ are hypoactive in CD45-neagative cells, but this difference is not observed if total cellular $p56^{lck}$ is assayed (Biffen et al., 1994). While cell-specific variation in subcellular localization is likely to be an important determinant in the regulation of PTK activity by CD45, accumulating data have provided new insights into the regulation of $p56^{lck}$ activity by tyrosine phosphorylation (Fig. 1-2). Notably, the autophosphorylation site of this PTK, Tyr-394, which is essential for activity (Abraham and Veillette, 1990), has been shown to determine the activation state of the kinase, regardless of the phosphorylation status of Tyr-505 (Hardwick and Sefton, 1997). Thus, in certain cellular contexts, the lack of CD45 results in hyperphosphorylation of both Tyr-394 and Tyr-505 of $p56^{lck}$, with the former modification upregulating kinase activity (D'Oro et al., 1996; Roach et al., 1997). It is not yet certain what factors regulate the balance of dephosphorylation of Try-394 and/or Tyr-505 of $p56^{lck}$ by CD45. It should be noted that that this revised model does not preclude Tyr-505 from functioning in a negative regulatory capacity. Rather, when phosphorylated, this residue is likely to interact with the SH2 domain of the kinase, preventing this module from being recruited

to cites of cellular phosphorylation as required for cellular activation (Luo and Sefton, 1992; Xu and Littman, 1993; Straus et al., 1996; Yamasaki et al., 1996).

F. Overview of T-cell Activation

A highly coordinated sequence of events accompanies T-lymphocyte activation induced either artificially, by Ab-mediated crosslinking of the TCR complex, or by Ag presented by an APC (Fig. 1-3). The nature of Ag presentation dictates that a T cell must be capable of recognizing, and being activated by, a specific class I/class II-peptide complex that is present on a presenting cell surface in conjunction with many other such complexes that are irrelevant to that particular T cell. Accordingly, several studies have demonstrated that T cells are capable of being activated by as few as 1 to 200 specific Ag complexes (Demotz et al., 1990; Christinck et al., 1991; Sykulev et al., 1996). The fact that so little Ag can promote an activation response is rationalized on a mechanistic level by the ability of a single peptide ligand to be serially engaged by multiple TCRs (Valitutti et al., 1995b; Beeson et al., 1996). In addition, the intracellular signal transduction modules that are assembled and activated provide significant amplification of signal strength.

As previously discussed, CD45 is capable of constitutively dephosphorylating CD4/CD8-associated pools of $p56^{lck}$. The status of $p56^{lck}$ phosphorylation at Tyr-505 is dictated by the balance between the opposing activities of CD45 and the intracellular PTK Csk, which acts upon the same site (Bougeret et al., 1996). In general, CD45 appears to be required to release the SH2 domain of this kinase from autoinhibition, thus

"priming" the kinase for full activation upon TCR triggering. While incomplete evidence has been obtained to suggest that the PTP activity of CD45 is required throughout T cell activation (Desai et al., 1993), others have argued that T cell activation cannot occur unless CD45 is excluded from the cell-to-cell interface, or "contact cap" (Shaw and Dustin, 1997). If exclusion of CD45 occurs (and this has yet to be verified experimentally), it may serve two functions, allowing closer juxtaposition of cell membranes by elimination of the bulky PTP and permitting an accumulation of phosphotyrosine-containing proteins in the intracellular space underlying the contact site. Contact cap formation is initiated by TCR and CD4/CD8 binding to class I/class II molecules and is further enhanced by other cell-surface adhesion molecules (Collins et al., 1994). CD4/CD8-mediated clustering of $p56^{lck}$ induces its activation by autophosphorylation at its positive regulatory site (Luo and Sefton, 1990), an effect that appears to require dimerization of the PTK (Duplay et al., 1996). Although other Src family kinases such as $p59^{fyn}$ share certain redundant functions with $p56^{lck}$ in T cells (Groves et al., 1996; van Oers et al., 1996), $p56^{lck}$ is necessary for optimal activation. Sequestration of $p56^{lck}$ from ligated TCRs has been shown to inhibit T cell proliferation (Maroun and Julius, 1994b), while T cells lacking this PTK exhibit marked defects in effector responses (Karnitz et al., 1992; al-Ramadi et al., 1996). In addition, mice deficient in $p56^{lck}$ exhibit gross defects in thymic maturation and T-cell activation (Molina et al., 1992).

Clustering and activation of $p56^{lck}$ sets in motion multiple branching signal transduction cascades. Activated $p56^{lck}$ mediates phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic tails of multiple

members of the CD3 complex, including CD3ζ (Iwashima et al., 1994). ITAM phosphorylation recruits additional Src family kinases and other SH2 domain-containing PTKs to the contact cap, including ZAP-70, which possesses two SH2 domains that interact in a coordinate manner with two phosphotyrosine residues in one of the three ITAMs in CD3ζ (Chan et al., 1992; Straus and Weiss, 1993). Interestingly, the SH2 domain of $p56^{lck}$ has been shown also to interact with CD3ζ and to be essential for processive phosphorylation of its ITAM motifs (Lewis et al., 1997), thus substantiating an activation-associated outcome dependent on release of the $p56^{lck}$ SH2 domain from autoinhibition. The identification of a human immunodeficiency syndrome associated with loss of ZAP-70 (Arpaia et al., 1994) and the generation of mice deficient in this PTK (Negishi et al., 1995) have helped establish an important role for ZAP-70 in T-cell development and activation. Src family kinases (particularly $p56^{lck}$) promote the activation of ZAP-70 by transphosphorylation following its recruitment to the contact cap (al-Ramadi et al., 1996; Yamasaki et al., 1996). Functional ZAP-70 is required for phosphorylation of guanine nucleotide exchange factor $p95^{vav}$, phosphorylation of adaptor proteins Shc and SLP-76, and activation of ERK2 MAP kinase, providing possible mechanisms whereby membrane proximal events activate pathways that converge on the nucleus to promote cell proliferation (Qian et al., 1996; Raab et al., 1997; Wu et al., 1997). In addition, $p56^{lck}$ has also been implicated in directly or indirectly promoting the tyrosine phosphorylation and activation of PLCγ (Weiss et al, 1991; Weber et al., 1992), which leads to initiation of Ser/Thr kinase- and Ca^{2+}-dependent signaling pathways. It should be noted that B-cell activation follows a remarkably similar course of events involving a related repertoire of signal transducing

molecules (Weiss and Littman, 1994). For example, in B cells, which minimally express $p56^{lck}$, CD45 regulates the related Src family PTK $p53^{lyn}$ (Katagiri et al., 1995).

Although the proximal signaling cascades associated with lymphocyte activation are understood in considerable detail, other aspects of the activation response are less completely characterized. For example, the cytoskeletal events that allow T cells to form a large, dynamic contact surface with APCs (Valitutti et al., 1995a) have not been elucidated. In addition, the mechanisms whereby homeostasis of signaling cascades is restored after Ag stimulation are poorly characterized, however it is likely that CD45 (Furukawa et al., 1994), Csk (Bougeret et al., 1996), and the PTP SHP-1 (Raab and Rudd, 1996) contribute to the downregulation of responses.

G. Regulation of CD45

Given the central importance of CD45 PTP activity in modulating activation responses, the question arises as to how its own enzymatic function is regulated. The idea that the PTP activity of CD45 may be modulated by reversible phosphorylation events, in a manner analogous to receptor PTKs, has received limited experimental support. An early study revealed that *in vitro* phosphorylation of CD45 by a variety of Ser/Thr kinases had no effect on its PTP activity (Tonks et al., 1990). In contrast, sequential *in vitro* phosphorylation of CD45 on Tyr followed by Ser (and not vice versa) resulted in enhanced activity toward one artificial substrate but not another (Stover and Walsh, 1994). Tyrosine phosphorylation of CD45 and induction of its enzyme activity have been observed upon co-expression in fibroblasts with the PTK $p50^{csk}$, however the

relevance of this overexpression study is uncertain (Autero et al., 1994). Treatment of T cells with the Ca^{2+} ionophore ionomycin has been found to inhibit CD45 PTP activity in a Ca^{2+}-dependent manner, but with relatively slow kinetics, a phenomenon that correlates with decreased Ser phosphorylation of CD45 (Ostergaard and Trowbridge, 1991). What is intriguing about this latter study is that it provides a possible mechanism for the global downregulation of CD45 by Ca^{2+}-sensitive Ser/Thr phosphatase(s) following cellular activation. Given that three *in vivo* sites of CD45 Ser phosphorylation have now been mapped (Kang et al., 1997), site-directed mutagenesis can be employed to clarify the ability of these sites to influence CD45 activity.

While reversible phosphorylation events may exert controls on CD45 activity, the high expression levels of the PTP and its apparent promiscuity raise the possibility that other mechanisms may be required to ensure that its activity its specifically directed. One hypothesis is that CD45 may interact in a dynamic manner with other cellular proteins that influence the ability of its PTP domain to act upon substrates. For example, CD45 may interact with proteins that restrict its membrane diffusion or subcellular localization and thus sequester its activity away from substrates. Alternatively, CD45-associated proteins may function as adaptors, bringing the PTP into close proximity with substrates. Finally, CD45 may be directed to dimerize, multimerize, or undergo conformational changes by other proteins and this may in turn influence the substrate accessibility of its PTP domain. These mechanisms are not mutually exclusive, and may be subject to developmental controls.

H. CD45-associated Proteins

Given the potential significance of CD45-associated proteins to the functions of CD45, it not surprising that intensive efforts have been made to identify such molecules. The list of proteins attested to be associated with CD45 is formidable, and includes: $p56^{lck}$ (Koretzky et al., 1993; Xu and Chong, 1995; Ng et al., 1996), $p59^{fyn}$ (Mustelin et al., 1992), CD4/CD8 (Mittler et al., 1991; Dianzani et al., 1992), TCR/Thy-1 (Volarevic et al., 1990), tyrosine phosphorylated CD3ζ (Furukawa et al., 1994), ZAP-70 (Mustelin et al., 1995), CD2 (Schraven et al., 1990), CD7 (Lazarovits et al., 1994), CD26 (Torimoto et al., 1991), CD100 (Herold et al., 1996), LFA-1 (Dianzani et al., 1992); rasGAP/Grb2/mSOS (Lee et al., 1996), fodrin (Lokeshwar and Bourguignon, 1992), and components of the B cell receptor complex (Justement et al., 1991; Brown et al., 1994). With the exception of the fodrin association, which was detected by coimmunoprecipitation in Triton X-100 detergent lysates, highly sensitive techniques such as immunofluorescent comodulation/cocapping, chemical crosslinking, fluorescence resonance energy transfer, and coimmunoprecipitation in Brij/digitonin lysates were employed to demonstrate these interactions. As a consequence, the specificity, stoichiometry, and *in vivo* relevance of these associations are uncertain. While residues 825-939 of CD45 are known to interact with fodrin (Iida et al., 1994), and the interaction of CD45 with tyrosine phosphorylated CD3ζ is stabilized by mutating the catalytic nucleophile in domain I of CD45 (Furukawa et al., 1994), it is not known by what means CD45 interacts with the other candidate associated proteins. More detailed studies are

required in a wider range of cell types to clarify the nature and functional relevance of the protein-protein interactions in which CD45 engages.

Recently a novel protein with a molecular mass of 30 kDa has been identified on the basis of its physical association with CD45 in a variety of cell types (Schraven et al., 1994; Takeda, 1994). The interaction of this transmembrane protein, named LPAP (lymphocyte phosphatase-associated phosphoprotein), with CD45 is mediated by the transmembrane segments of the two proteins (Cahir McFarland and Thomas, 1995; Bruyns et al., 1996). LPAP lacks any recognizable primary sequence motifs and its true function remains elusive. LPAP protein and, remarkably, mRNA, are unstable in cells lacking expression of CD45 (Schraven et al., 1994), and a recent study indicates that LPAP associates with CD45 immediately upon biosynthesis (Cahir McFarland et al., 1997). In contrast, CD45 does not require LPAP for its stability, since LPAP is not expressed in a variety of CD45-positive cells, including terminally differentiated granulocytes and monocytes (Shimizu et al., 1997).

I. Puzzling Facets of CD45 Biology

Although CD45 has been intensely studied over the past decade and many advances have been made, questions remain regarding many aspects of its biology. One of the most puzzling issues surrounds the function of the extracellular domain of CD45. The relevance of the strictly regulated expression of CD45 isoforms and the microheterogeneity conferred by *N*- and *O*-linked glycosylation remain completely unresolved. Although it seems reasonable to propose that different CD45 isoforms may

selectively recognize specific ligands, no molecule has yet been identified that interacts exclusively with the CD45 ectodomain. In addition, it is not known whether CD45 functions exclusively to regulate Src family kinases. It is possible that CD45 may be important for downregulating activation responses, however, such a function would be masked in CD45-deficient cells due to defects in proximal signaling pathways. It is also possible that CD45 performs general functions in a wide variety of hematopoietic cell types. The fact that CD45 can associate with fodrin, for example, raises the possibility that it may regulate the cytoskeleton. Finally, it remains uncertain what proteins CD45 specifically associates with in different cell lineages at different developmental stages, and whether these interactions may influence its function.

J. Rationale and Objectives

It is hypothesized that CD45 is functionally coupled to signaling cascades that are modulated when its external domain is engaged *in vivo* by mechanisms that have not yet been elucidated. The first objective of the present study is to utilize an *in vitro* model to examine signal transduction events and functional outcomes associated with CD45 ligation.

Since CD45 may engage in protein-protein interactions (including ligand interactions) that regulate previously uncharacterized facets of its function, the second aim of this study is to identify proteins that specifically interact with CD45 and determine whether these interactions occur in a broad or restricted range of cell types.

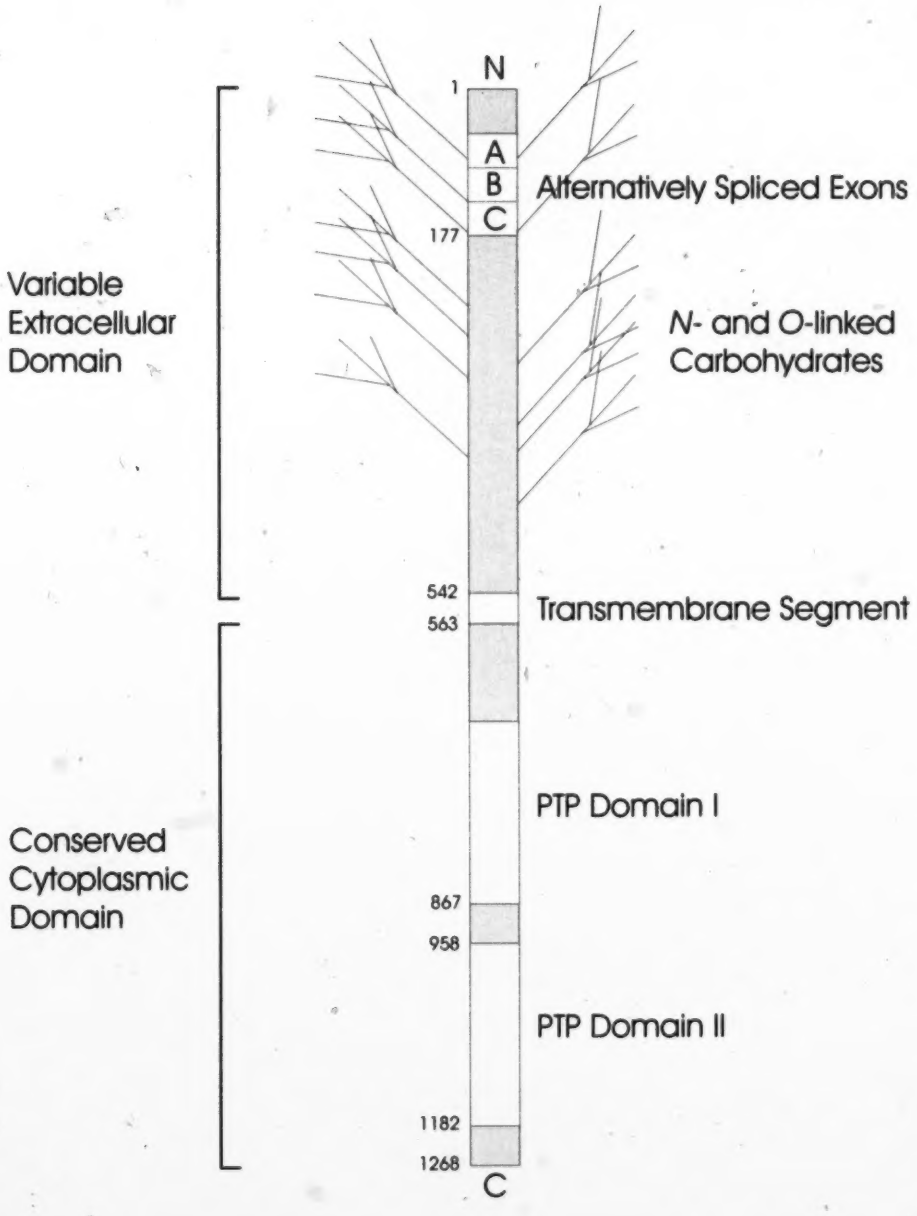

FIGURE 1-1. Schematic diagram of CD45 protein-tyrosine phosphatase. The extracellular domain of CD45 is highly *N*-glycosylated, and the amino-terminal region encoded by the variably expressed exons is rich in *O*-glycosylations. The cytoplasmic domain contains two repeated PTP homology domains, however the activity of membrane-distal domain II has not been convincingly demonstrated. Amino acid residues are numbered. (Adapted from Trowbridge et al., 1991).

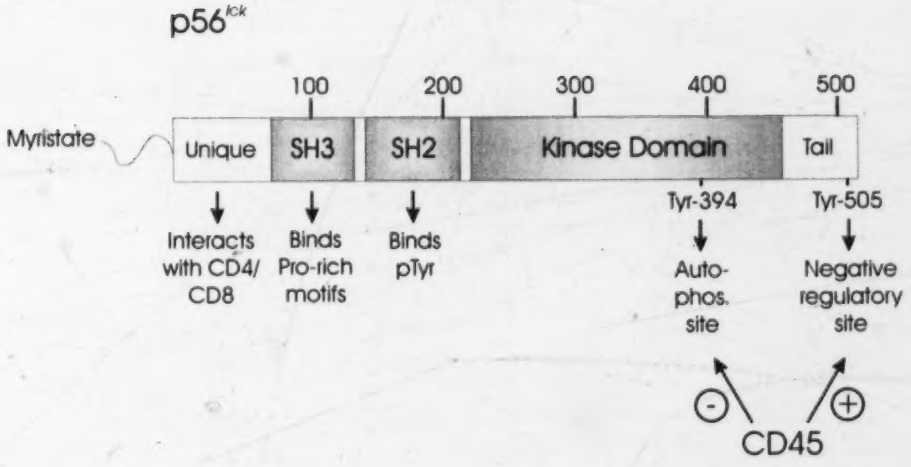

FIGURE 1-2. Dichotomous role of CD45 in regulating p56^{lck} activity. The structure of Src-family PTK p56^{lck} is shown, along with sites of regulation by CD45 PTP. Dephosphorylation of carboxyl-terminal Tyr-505 by CD45 frees the PTK from an autoinhibitory intramolecular interaction with its SH2 domain, allowing it to assume an open configuration in which the kinase and SH2 domains are fully accessible. CD45 is also capable of directing its activity to Tyr-394, the positive regulatory autophosphorylation site of p56^{lck}. Dephosphorylation at this site results in downmodulation of PTK activity.

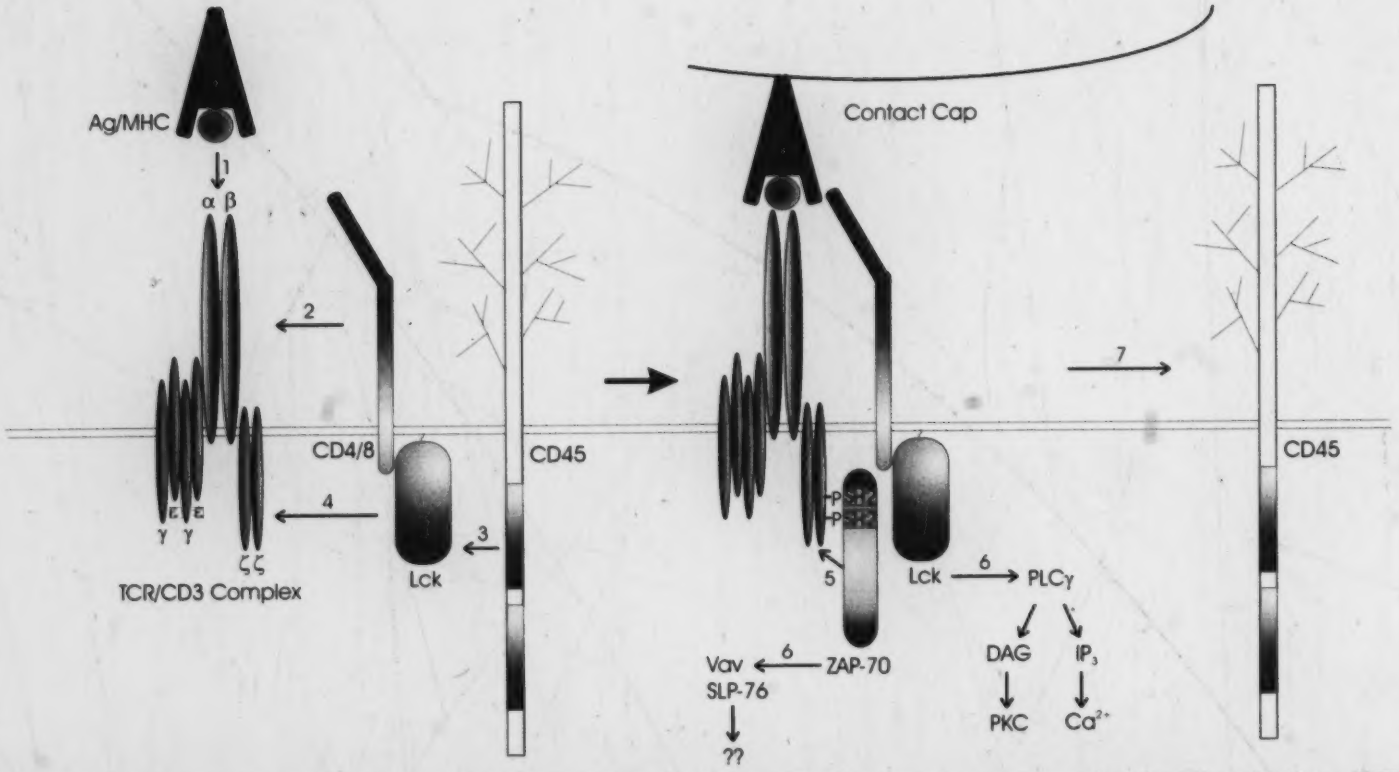

FIGURE 1-3. Simplified overview of proximal intracellular signal transduction events accompanying T-cell activation. TCR α and β chains interact with Ag presented in the context of class I/class II MHC proteins (*1*) as does CD4/CD8, leading to recruitment of CD4/CD8-associated $p56^{lck}$ (*2*) that is maintained in a semi-activated state by CD45 (*3*). The $p56^{lck}$ kinase mediates the phosphorylation of ITAMs within the ζ chain of the TCR/CD3 complex (*4*), permitting recruitment of ZAP-70 via interaction of its tandem SH2 domains (*5*). Activated $p56^{lck}$ and ZAP-70 function to trigger additional downstream effectors via pathways that have not been fully elucidated (*6*). Meanwhile, CD45 may be excluded from the contact cap formed at the cell-to-cell interface (*7*), allowing for the accumulation of phosphotyrosine in this region.

CHAPTER II

MATERIALS AND METHODS

Tumor cell lines. The mouse T-lymphoma cell lines SAKRTLS 12.1 (SAKR) and BW5147 (BW), CD45-negative variants of these lines (SAKR/CD45$^-$ and BW/CD45$^-$), and a revertant of BW/CD45$^-$ expressing a truncated form of CD45 (BW/rev) were obtained from Dr. Robert Hyman (Hyman and Trowbridge, 1981; Hyman et al., 1982). Dr. Hyman also supplied the AKR thymoma line. The PHA^{R}2.7 cell line, provided by Dr. Ian Trowbridge, is a BW-derived mutant deficient in α-glucosidase II (Trowbridge and Hyman, 1978). The EL4 T-cell lymphoma was obtained from ATCC, as were B-cell hybridomas NS-1 and LK35. CD45$^+$ and CD45$^-$ YAC-1 T-lymphoma cells were provided by Dr. Jonathon Ashwell (Volarevic et al., 1992). T-helper hybridomas A1.1 (Shi et al., 1990) and AODH7.1 (Kappler et al., 1981) were obtained from Dr. Lianfa Shi and Dr. John Kappler, respectively. All mouse hybridomas and lymphomas were grown in DMEM (Life Technologies) supplemented with 8% DCS (HyClone). The human leukemic Jurkat T-cell line, the CD45-deficient variant J45.01 (Koretzky et al., 1991), and the p56^{lck}-deficient variant J.CAM1.6 (Straus and Weiss, 1992) were obtained from ATCC and cultured in RPMI (Life Technologies) containing 10% FCS (HyClone).

Cloned CTL and non-transformed T-cell lines. The murine CD8$^+$ CTL clones AB.1, 11, and 10/1 have been described previously (Blakely et al., 1987; Kane et al.,

1989). Clone cells were maintained with weekly Ag stimulation by culturing with irradiated allogeneic spleen cells prepared by Nancy Berg, and experiments were conducted on days 4 to 6 post-stimulation. Clone cells were grown in clone medium consisting of RPMI supplemented with 8% FCS, 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), 100 U/ml penicillin-streptomycin (Life Technologies), and 5 x 10^{-2} mM 2-ME. IL-2-containing supernatant from TPA-induced EL4 cells was added to the cell cultures at 15 U/ml twice weekly. Clone 2C, an Ag-independent CTL clone, was maintained in the absence of stimulator cells in a manner otherwise identical to that described above. The T-cell line CTLL-2 and a $p56^{lck}$-deficient variant of this line (Karnitz et al., 1992), generously provided by Dr. Robert Abraham, were also maintained in IL-2 containing clone medium in the absence of antigenic stimulation.

CD45-transfected fibroblasts and other fibroblast cell lines. Dr. Pauline Johnson provided the mouse ψ2 cells infected with pARV-1 retroviral constructs expressing individual isoforms of CD45 (Johnson et al., 1989b). These cells were grown in DMEM containing 8% DCS and 2 mM L-glutamine. The A431 human epidermal carcinoma cell line, obtained from ATCC, was maintained in the same manner. Fibroblast cells were harvested by resuspension in ice-cold Versine (Life Technologies).

Ex vivo mouse cells. *Ex vivo* thymocytes and splenocytes were isolated from young (~8-16 week-old) mice of various strains. Rita Marcott provided the BALB/c cells, including Ficoll-enriched splenocytes.

Metabolic labeling, surface iodination, and surface biotinylation. For metabolic labeling with [^{35}S]Met, 10^7 cells/ml were incubated for 4 h with 0.2 mCi/ml of Tran^{35}S-label (ICN) in Met-free RPMI medium containing 10% dialyzed DCS. Cells were washed 3 times in D-PBS (Life Technologies) before lysis. For radio-iodination of surface proteins, cells were harvested, washed, and resuspended in 5 mM glucose-PBS at a density of 2 x 10^7/ml. The labeling reaction was initiated by addition of 1 mCi/ml ^{125}I along with 0.25 U of glucose oxidase and 1.25 U of lactoperoxidase. After a 10-min incubation at 22°C, the reaction was quenched by addition of 2 ml of cold PBS. Cells were washed in PBS in preparation for lysis. Cells were prepared for surface biotinylation by washing twice in D-PBS and resuspending at 4 x 10^7 cells/ml in D-PBS containing 120 μg/ml Sulfo-NHS-Biotin (Pierce). Labeling was allowed to proceed for 10 min at room temperature and was quenched by incubation with an equal volume of culture medium for 5 min at 4°C. The supernatant was discarded and the quenching step was repeated. Cells were then washed 3 times in cold D-PBS in preparation for lysis.

Monoclonal antibodies. The monoclonal antibodies (mAbs) I3/2.3 (I3/2), obtained from Dr. Ian Trowbridge, and M1/9.32 HL2 (M1/9), obtained from ATCC, recognize pan-specific determinants in the ectodomain of mouse CD45 (Trowbridge, 1978; Springer et al., 1978). GAP 8.3, a pan-specific mAb recognizing the extracellular domain of human CD45, was obtained from ATCC, as was M1/42.3.9.8 (M1/42), a mAb directed against a haplotype-independent epitope of mouse class I MHC molecules. The above mAbs were purified by ammonium sulfate precipitation and directly coupled to

CNBr-activated Sepharose-4B (Sigma). IM7.8.1 (anti-CD44), M17/5.2 (anti-LFA-1) and R17 217.1.3 (R17; anti-transferrin receptor) were obtained from ATCC. The mAbs PY72.10.5 (PY72; anti-phosphotyrosine) and 145-2C11 (2C11; anti-CD3ε) were obtained from Dr. Jeffrey Bluestone and Dr. Bart Sefton, respectively (Cooper et al., 1983; Leo et al., 1987). Hybridoma cells secreting mAb were cultured in Protein Free Hybridoma Medium-II (Life Technologies) and supernatants were concentrated by ammonium sulfate precipitation followed by dialysis. Purity of mAbs was verified by SDS-PAGE, and BCA assays (Pierce) were performed to establish protein concentrations.

Polyclonal antisera. The polyclonal antiserum to the recombinantly expressed cytoplasmic domain of mouse CD45 has been described (Ostergaard et al., 1989). Rabbit antiserum directed against a peptide corresponding to the 33 carboxyl-terminal residues of $p56^{lck}$ was generated by standard procedures by Dr. Hanne Ostergaard. Affinity-purified anti-phosphotyrosine rabbit antiserum was obtained from Upstate Biotechnology. Antisera 80.1 and 80.2, specific for residues 112-258 and 437-514 of mouse glucosidase II β-subunit (GII_β) were generated as described below. The GII_β-reactive antisera were subjected to ammonium sulfate precipitation. Antiserum 80.2 was further purified by two cycles of absorption to immobilized glutathione S-transferase (GST) for use in the mapping study in Chapter VII. Preimmune sera from the above rabbits were obtained for control experiments.

Flow cytometry. A total of 1.5 x 10^6 cells/tube were resuspended in IF buffer (0.1% FCS, 0.02% sodium azide, PBS) containing 20 μg/ml primary Ab. Cells were incubated at 4°C for 30 min, washed twice in cold IF buffer, and incubated for 30 min with goat anti-rat^{FITC} secondary Ab (Jackson ImmunoResearch) at a 1:50 dilution in IF buffer. Cells were then washed 3 times in IF buffer and resuspended in 1% paraformaldehyde for analysis by flow cytometry. The mean channel fluorescence intensity of stained cells was assessed by FACScan using LYSIS II software (Becton Dickinson). Cells incubated with second Ab alone were used as a baseline control to correct for background staining.

Antibody immobilization. The mAbs were diluted to 20 μg/ml in PBS and immobilized to plastic 96-well flat-bottom microtiter plates (Nunc) by adding 50 μl/well and incubating for 90 min at 37°C. For co-immobilization of anti-CD45 with anti-CD3, I3/2 was added to wells at 20 μg/ml along with either 1.6 μg/ml 2C11 (2C11^{lo}) or 16 μg/ml 2C11 (2C11^{hi}). Following the Ab incubation, the wells were blocked with 2% BSA (Boehringer Mannheim) in PBS for 90 min at 37°C and then were washed 3 times with PBS.

Morphologic change assays. Cells were washed in D-PBS and resuspended in RPMI or D-PBS containing 0.1% FCS. Microtiter plates prepared with immobilized mAb were seeded at a density of 5 x 10^4 cells/well and placed in a 37°C CO_2 incubator. Morphologic change was assessed at various timepoints by counting the number of cells at the center of each well exhibiting a flattened phenotype (accompanied by a loss of

membrane refractility) of the total number of cells as revealed by inverted light microscopy. A minimum of 100 cells/well were counted. Experiments were performed in duplicate, and the percent morphologic change is expressed as an average of wells from a representative experiment. Photomicrography at ×200 magnification was conducted using a Zeiss inverted microscope attached to a UVP video imaging system.

Chemical inhibitors. Stock solutions of cytochalasin D (Sigma) and cytochalasin E (Sigma) were prepared at 1 mM in ethanol, and herbimycin A (Life Technologies) was dissolved at 1 mg/ml in DMSO. Cells were pretreated with inhibitors or with carrier alone for 15 min at room temperature before being transferred to microtiter wells. The inhibitors remained present throughout the entire assay period.

Preparation of lysates from cells undergoing mAb-induced changes in morphology. Cells were washed in D-PBS and resuspended in 0.1% FCS-RPMI. After warming to 37°C, 40 μl of this suspension was added to mAb-absorbed microtiter wells at a final density of 1.0-1.5 x 10^5 cells/well. Cells were allowed to settle at 37°C and were lysed at various time intervals by adding 40 μl of a 2X stock of reducing SDS sample buffer (1X = 2% SDS, 5% 2-ME, 2.5% glycerol, 0.02% bromphenol blue, and 68 mM Tris, pH 5.7) and boiling immediately. For preparation of detergent lysates for immunoprecipitation studies, cells were incubated in mAb- or BSA-coated wells as described above. After 30 min, cells were lysed in a final volume of 0.5% Triton X-100, 1 mM $NaVO_4$, 0.85 mM EDTA, 150 mM NaCl, 20 mM PO_4, pH 7.4. Lysates from 20 wells were pooled for each immunoprecipitation condition.

Preparation of lysates from cell stimulated with TPA or ionomycin. Cells were stimulated with the phorbol ester TPA at a concentration of 0.1 μg/ml or with the calcium ionophore ionomycin at 0.4 μM for 10 min at 37°C. Cells were solubilized in lysis buffer (0.5% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.6) supplemented with 1 mM $NaVO_4$ as a PTP inhibitor.

Preparation of lysates from unstimulated cells. Cells were washed 3 times in D-PBS and resuspended in ice-cold lysis buffer (0.5% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.6) at a concentration of 4-5 x 10^7 cells/ml.

Immunoprecipitations. Detergent lysis was allowed to proceed on ice for 20-30 min, after which postnuclear extracts were prepared by 5-min centrifugation at 14,000 rpm in a cooled microfuge. For immunoprecipitation with Sepharose-4B-conjugated mAbs, the beads were first blocked by incubation with 3% BSA-PBS for 30 min at room temperature. For immunoprecipitations employing rabbit antisera, lysates were incubated for 30-60 min on ice with 5 μl of antisera, followed by addition of Protein A-Sepharose beads (Pharmacia Biotech). For immunoprecipitation of CD44, 15 μg of IM7.8.1 was added to lysates for 30 min followed by 5 μl rabbit anti-rat IgG antiserum (Jackson ImmunoResearch Laboratories) for 30 min before capture by Protein A beads. In most experiments, immunoprecipitations were allowed to proceed for 1-3 h on an orbital rotator in the coldroom. Beads were then washed 3 to 8 times with cold lysis buffer. In some experiments, particularly those involving detection of proteins by total staining

procedures, beads were first washed 3 to 5 times with high salt lysis buffer (lysis buffer containing 0.5 M NaCl), followed by 1 to 3 washes in standard lysis buffer. For immunoprecipitation of $p56^{lck}$, immune complexes were washed 4 times with RIPA buffer (1% NP-40, 1% DOC, 0.1% SDS, 150 mM NaCl, 10 mM PO_4, pH 7.2). After the final wash, proteins were released by boiling in 1X reducing SDS sample buffer.

Polyacrylamide gel electrophoresis and membrane-transfer of proteins. Unless otherwise indicated, reduced and denatured proteins were loaded onto 7.5% polyacrylamide gels for electrophoretic separation by the Laemmli method (Laemmli, 1970). If appropriate, proteins were transferred to Immobilon-P (Millipore) in 20% methanol, 20 mM Tris, and 96 mM glycine for 2 to 3 h at 60V. The molecular masses of proteins were estimated by comparison to the mobilities of known standard proteins (Bio-Rad and Sigma). Modified conditions were used in experiments involving CNBr-digested proteins, as detailed below.

Total protein staining of gels and membranes. After transfer to Immobilon-P, proteins were stained with 0.1% India ink in 0.1 M NaH_2PO_4, 0.15 M NaCl, and 0.3% Tween-20 for at least 24 hours. Staining of protein gels by the silver stain method or with Coomassie Brilliant Blue R-250 dye (Bio-Rad) were carried out according to standard procedures.

Western blotting. In preparation for Western blot analysis, membranes were blocked overnight in 4% BSA or 4% skim milk powder in membrane wash buffer (0.1%

Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.6). Probing of blots with each of the reagents described below was allowed to proceed for approximately 1 h in blocking buffer. Biotinylated proteins were detected by incubation with Streptavidin^{HRP} (Boehringer Mannheim) at 1:30,000. For detection of anti-phosphotyrosine, the mAb PY72 was used at 1 μg/ml, followed by goat anti-mouse^{HRP} (Jackson ImmunoResearch Laboratories) at 1:20,000. Where indicated, tyrosine phosphoproteins were also identified by probing with anti-phosphotyrosine antiserum at 1 μg/ml, followed by goat anti-rabbit^{HRP} (Jackson ImmunoResearch Laboratories) at a dilution of 1:25,000. For detection of CD45, the rabbit anti-95K serum was used at a dilution of 1:7500. The GII_{β}-specific reagents anti-80.1 and anti-80.2 were utilized at 1:10,000 to 1:15,000. Membranes labeled with rabbit antisera were incubated with Protein A^{HRP} secondary reagent (Pierce) at 1:30,000. Labeled bands were identified by exposing the blots to Reflection Autoradiography Film (DuPont-NEN) following addition of ECL detection reagents (DuPont-NEN). To allow certain blots to be sequentially probed with distinct primary reagents, a stripping reaction was carried out between each blotting experiment to remove bound Abs. Blots were incubated at 56°C for 30 min in stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris, pH 6.7) followed by extensive washing in membrane wash buffer. Blocking of membranes was then carried out as usual. The colorometric blotting experiment in Chapter IV was conducted using the Immun-Blot Alkaline Phosphatase Assay Kit (Bio-Rad).

In vitro reconstitution of binding assay. I3/2 immunoprecipitates were prepared from SAKR cells, unless otherwise stated. Beads were washed twice with elution buffer

(0.5% DOC, 20 mM Tris, pH 7.6) to remove CD45-associated proteins and twice with lysis buffer. Alternatively, immunoaffinity purified CD45 (obtained as described below) was incubated with I3/2 beads in 0.5 ml lysis buffer for 1 h. Unless otherwise noted, the CD45 beads were incubated 1-3 h with 1 ml of detergent-solubilized extract from 5 x 10^7 SAKR/CD45$^-$ cells. Immune complexes were washed and processed for SDS-PAGE. In the case of the reconstitution of binding experiment in Chapter IV involving individual CD45 isoforms immunoprecipitated from ψ2 cells, the elution buffer wash was omitted.

Reconstitution of CD45 binding to p116 from cell membranes. SAKR/CD45$^-$ cells were suspended at 5 x 10^7/ml in PBS containing 0.2 mM PMSF (Pierce) as a protease inhibitor. Nitrogen cavitation was carried out for 6.5 min at 500 psi, at which point the membranes of 95% of the cell population were disrupted as confirmed by uptake of trypan blue stain. The nuclear fraction was eliminated by a 15-min spin at 3,600 *x g* in a RC5C centrifuge (Sorvall Instruments, DuPont). The supernatant was centrifuged in for 15 min at 22,000 *x g* and the resulting soluble fraction, containing cytosolic proteins, was placed on ice. The pellet containing cell membranes and cytoskeletal components was reconstituted at 1.6 mg/ml in lysis buffer. Half of this material was set aside (fraction 1) and the other half was subjected to an additional high speed centrifugation step for 45 min at 37,000 rpm in the 70i rotor of an L8-80M ultracentrifuge (Beckman Instruments). This supernatant, labeled fraction 2, corresponds to the membrane faction depleted of insoluble components (i.e. cytoskeletal proteins). Cytosolic and membrane fractions were subjected to I3/2 immunoprecipitation in the presence or absence of approximately 10 μg of purified CD45. Beads mixed with

detergent-solubilized membranes were prepared for SDS-PAGE by washing 5 times in high salt lysis buffer and 3 times in regular lysis buffer. Beads treated with cytosolic proteins in the absence of detergent were washed 8 times in PBS.

Endoglycosidase treatment. For the work described in Chapter IV, Endo F digestions were performed by incubating immune complexes overnight at 37°C with 0.3 U of Endo F (Boehringer Mannheim) in 50 µl of 20 mM EDTA, 1% 2-ME, and 0.25 M sodium acetate, pH 6.5. The immunoprecipitates were washed 3 times in lysis buffer, resuspended in reducing SDS sample buffer, and subjected to SDS-PAGE. For the experiments detailed in Chapter V, immunoprecipitates were incubated with a 60-µl reaction mix consisting of 0.5 U Endo F in 20 mM EDTA, 0.2 M sodium acetate, pH 6.0, or 10 mU Endo H (Boehringer Mannheim) in 0.2 M sodium acetate, pH 6.0. Digests were allowed to proceed with constant agitation for 16 h at 37°C. Mock digestions were carried out in an equivalent volume of glycosidase-free digestion buffer. Immune complexes were either boiled immediately in reducing SDS sample buffer or were subjected to additional washing steps before being either reduced and denatured or tested in the reconstitution of binding assay.

Purification of CD45 and CD45-associated proteins. Post-nuclear extracts from 1-2 x 10^{10} viable SAKR or SAKR/CD45$^-$ cells were prepared under the same conditions used for preparation of small-scale extracts, with the exception that the nuclear fraction was separated by centrifugation for 10 min at 12,000 *x g*. Nuclear-free supernatants were passed through 0.45-µm filters (Costar), and applied overnight to affinity columns

containing 10-ml bed volumes of Sepharose-4B-I3/2 or -M1/42. The columns were washed with 200-300 ml of high salt (0.5 M NaCl) wash buffer followed by approximately 150 ml of lysis buffer. Elution buffer (0.5% DOC, 20 mM Tris, pH 7.6) was applied and 1-ml fractions were collected, saving 40 µl from each for electrophoretic analysis. Purified CD45 was subsequently eluted from the column by addition of 0.1% NP-40, 0.5 M NaCl, 50 mM sodium acetate, pH 4.0, followed by immediate neutralization of pH. Fractions were analyzed by SDS-PAGE revealing CD45 as the only species visible by silver staining.

Microsequence analysis. I3/2 immunoaffinity column fractions positive for the 80-kDa and 116-kDa CD45-associated proteins were pooled and concentrated using Ultrafree-MC Filters (Millipore). Concentrated proteins were separated on preparative gels with the addition of 0.1 mM sodium thioglycolate to the cathode buffer to minimize chemical blockage of amino-reactive groups. After transfer to Immobilon-P, proteins were stained for 45 s in 0.025% Coomassie Brilliant Blue R-250, 40% methanol, and 5% acetic acid. Destaining was carried out in the identical solvent, minus the dye. Protein bands corresponding to the 80-kDa and 116-kDa proteins were excised for solid phase amino-terminal microsequence analysis by the Alberta Peptide Institute (Edmonton, AB) or cut into mm-sized fragments for overnight digestion with 30 mg/ml of CNBr in 70% formic acid. The digestion buffer was then removed and concentrated in a vacuum centrifuge (Savant Instruments). To elute membrane-bound peptides, elution solvent (0.1 mM lysine, 0.1 mM thioglycolic acid, 0.2% trifluoroacetic acid, 70% isopropanol) was added for 2.5 h. Eluted peptides were pooled and concentrated together with the previous

supernatants, and the elution step was repeated. After drying, cleavage products were washed in deionized water and dried again. The peptides were then boiled in reducing SDS sample buffer and loaded onto 16.5% Tris-Tricine Mini-PROTEAN II gels (Bio-Rad). Electrophoresis was carried out using a cathode buffer composed of 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, and 0.1 mM sodium thioglycolate and an anode buffer of 0.2 M Tris, pH 8.9. Peptide molecular weight protein standards (Bio-Rad) were run alongside the samples. Electrophoresis was carried out for 2 h at 100 V, and proteins were transferred to Immobilon-P^{SQ} (Millipore Corp.) for 1 h at 100 V. Coomassie Blue staining was performed by the same method used earlier in the procedure. After extensive washing in deionized water, the protein bands were excised for microsequence analysis by the Alberta Peptide Institute.

cDNA cloning of glucosidase II α-subunit. Degenerate oligonucleotides corresponding to regions of protein sequence obtained for the 116-kDa protein were synthesized (as were all oligonucleotides) by the University of Alberta Department of Biochemistry DNA Sequencing Facility. The degenerate primers were screened by PCR for amplification of DNA fragments from SAKR cDNA. To prepare the cDNA, SAKR RNA was first extracted using the Micro RNA Isolation Kit (Stratagene). Poly(A)$^+$ transcripts were purified by oligo(dT) chromatography using the Poly(A) Quik mRNA Isolation Kit (Stratagene). Reverse transcription was performed using SuperScript II (Life Technologies) and random primers (Stratagene). Two primers, 5'-tt(ct)ga(ag)ca(ct)ca(ag)(ac)g(ag)gc-3' (a sense oligonucleotide corresponding to internal peptide sequence FEHQRA), and 5'-at(agct)gg(ag)tc(ag)tc(ct)ttcat-3' (an antisense

oligonucleotide corresponding to internal peptide sequence MKDDPI), were found to amplify a 1.9-kb DNA fragment. The optimal parameters for this reaction were 30 cycles of 20 s at 94°C, 2.5 min at 55°C, and 2 min at 72°C, followed by a final 8-min extension at 72°C. Oligonucleotides were added at a final concentration of 0.25 μM each multiplied by the degeneracy factor to a 50-μl reaction containing 2.5 U Taq polymerase (Promega) in 2 mM Mg^{2+}, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris-HCl, pH 9. The 1.9-kb amplicon was cloned by the TA overhang method into the pCRII vector (Invitrogen), and partial sequencing was performed to confirm the presence of nucleotide sequence at the 5' and 3' ends of the insert in agreement with the amino acid microsequence data. All DNA sequences were determined by the dideoxy chain termination method on an ABI 373A automated sequencer (Applied Biosystems) by the University of Alberta Department of Biochemistry DNA Sequencing Facility. The 1.9-kb insert was excised, gel-purified with Geneclean III (Bio 101), and labeled with 5'-α[^{32}P]dCTP (Amersham) by random priming using the High Prime DNA Labeling Kit (Boehringer Mannheim). The labeled probe was depleted of unincorporated nucleotides by chromatography on Sephadex G-50 (Pharmacia) and used to screen a Uni-ZAP XR oligo(dT)-primed mouse EL4 T-cell cDNA library as per the instructions of the manufacturer (Stratagene). Briefly, phage-infected XL1-Blue cells were grown on NZB agar plates at 42°C for 4-5 h. Phage DNA from plaque lifts onto nitrocellulose membranes (Stratagene) was released and denatured in 0.5 M NaOH and 1.5 M NaCl, and then cross-linked to the membrane by baking at 80°C for 2 h. Membranes were incubated in prehybridization buffer consisting of 2X PIPES, 50% ultrapure formamide (Life Technologies), 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA for at least

2 h at 42°C. The labeled probe was heat denatured and added immediately to the prehybridization buffer. The labeling reaction was allowed to proceed overnight at 42°C in a hybridization incubator (Tyler Research Instruments). Membranes were washed in 0.1X standard sodium citrate containing 0.1% SDS twice at room temperature, followed by 2 washes of 15 min at 67°C, before exposing to XAR film (Kodak) with an intensifying screen at -70°C. Positive phage excised from the original plates were subjected to further rounds of screening until all plaques tested positive. Phagemid excision was then carried out by co-infection of XL1-Blue cells with the ExAssist helper phage (Stratagene). Phagemids obtained in the supernatant fraction were used to infect *E. coli* SOLR (Stratagene), and the amplified pBluescript plasmids were extracted by miniprep (QIAGEN). Two independently isolated but apparently identical clones encoding glucosidase II α-subunit (GII_α) were rescued from a screen of approximately 300,000 colonies. The clones, measuring 2.9-kb in length, were found to be incomplete after sequencing. To isolate the 5' end of the cDNA, a cDNA library was constructed with the Marathon cDNA Amplification kit (CLONTECH) using 0.7 μg SAKR poly(A)$^+$ RNA as starting material. Following synthesis of first and second cDNA strands, an oligonucleotide adaptor provided with the kit was ligated to all molecules in the library. Two PCR-RACE reactions were carried out using specific antisense primers designed to span nucleotides 1586-1609 (first round) and 231-260 (second round), in conjunction with the sense-oriented adapter primer (AP1) supplied with the kit. The high fidelity Advantage cDNA Polymerase Mix (CLONTECH) was used in these reactions under the following cycling conditions: 1 round of 1 min at 94°C (to denature the hot-start Ab) followed by 28 rounds of 30 s at 94°C, 30 s at 64°C, and 3 min at 68°C. Products of

approximately 1.6 kb (first round) and 0.3 kb (second round) were TA cloned directly from the reaction mixes. Positive clones containing sequences overlapping those of the incomplete EL4-derived cDNA were fully sequenced in both directions. Finally, the entire open reading frame (ORF) of GII_α was PCR-amplified from SAKR using a primer pair spanning nucleotides 1-26 and 3065-3091, cloned into pCRII, and sequenced by primer walking.

cDNA cloning of glucosidase II β-subunit. The entire ORF of human homologue 80K-H (Sakai et al., 1989) was cloned by RT-PCR from the A431 cell line using primers spanning nucleotides 129-149 and 1718-1739. After confirming its identity by restriction analysis, the 1.6-kb amplicon was radiolabeled and used to screen the EL4 cDNA library by cross-species hybridization. The protocol used was identical to that described above for cloning of GII_α, with the exception that post-hybridization washes were carried out at 60°C rather than 67°C. Five positive clones were rescued from a screen of approximately 300,000 plaques, each with inserts of different sizes corresponding to portions of the 3' end of mouse glucosidase II β-subunit (GII_β) mRNA, as revealed by partial sequencing. The longest of these, clone 80-5 (1.75 kb), was completely sequenced along both strands. The remaining 0.6-kb 5' end of the cDNA was cloned by PCR-RACE as outlined above for GII_α, using a gene-specific primer spanning bases 603-636. In addition, the entire ORF was sequenced following RT-PCR amplification from SAKR cDNA using the primer pair 70-97 and 1681-1707. 3' PCR-RACE clones of GII_β, one of which was examined for evidence of alternative splicing in

Chapter VI, were derived using a gene-specific primer spanning 545-567 and reaction conditions identical to those used to obtain 5' PCR-RACE clones.

Generation of GST-fusion proteins. Flanking primers were designed to introduce an upstream *Eco*RI restriction site and a downstream in-frame stop codon followed by a *Xho*I restriction site. Inserts were amplified using the high fidelity Advantage cDNA Polymerase Mix by PCR from GII_β cDNA clones obtained as described above. For most reactions, the thermokinetic parameters were 1 cycle of 1 min at 94°C followed by 25-30 cycles of 30 s at 94°C, 1 min at 54°C, and 50 s at 68°C. PCR products were cloned into the pGEX-4T-3 GST expression vector (Pharmacia). To reduce the number of false positives, the vector was dephosphorylated by calf intestinal phosphatase (Promega) and gel purified (Bio 101) prior to the ligation reaction. Ligation products were used to transform *E. coli* strain JM105. Positive clones were identified by plasmid purification followed by restriction analysis and/or by screening the bacteria for expression of recombinant protein. GST-fusion protein expression was induced in log-phase bacterial cultures by addition of 0.1 mM IPTG for 2 h. Bacteria were lysed by sonication in PBS containing 1 mM Pefabloc SC (Boehringer Mannheim) as a protease inhibitor, and soluble GST-fusion proteins were purified by batch elution from glutathione-agarose matrix (Sigma) according to the instructions provided with the vector. The elution buffer employed was 10 mM reduced glutathione (Sigma) in 50 mM Tris, pH 8.0. Following extensive dialysis against PBS, protein concentrations were measured by standard BCA assay. Recombinant proteins were adjusted to 0.5 mg/ml and

stored at -70°C for later use. Purity of fusion proteins was assessed by subjecting samples to SDS-PAGE on 10% gels followed by Coomassie Blue staining.

Generation of polyclonal antisera to GII$_\beta$. A highly pure GST-fusion protein comprising amino acid residues 112-258 of GII$_\beta$ (where residue 1 follows the site of signal peptide cleavage) was injected into a rabbit via intramuscular and subcutaneous routes. Injections were administered by University of Alberta Health Sciences Laboratory Animal Services. Protein for the first injection was mixed with Freund's Complete Adjuvant (Sigma), while material for subsequent injections was prepared in a base of Freund's Incomplete Adjuvant (Sigma). Injections were carried out on a bi-monthly schedule until a maximum response was achieved. For the final two injections, GST was first removed from the recombinant protein by thrombin cleavage as per the instructions supplied with the expression system. The antiserum thus obtained was designated anti-80.1. The same strategy was employed to generate a second antiserum, designated anti-80.2, against a GST-fusion protein spanning carboxyl-terminal amino acid residues 437-514 of GII$_\beta$, although in this case the thrombin cleavage step was omitted.

Genomic DNA sequencing. Genomic DNA was extracted from 2 x 10^7 SAKR cells using the QIAamp Tissue Kit (QIAGEN). Genomic DNA at 2 to 4 μg/ml was amplified in PCR reactions catalyzed by 50 U/ml Taq polymerase (Promega) in the presence of 1.5 mM Mg^{2+}, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris-HCl (pH 9), 0.2 mM each dNTP, and 0.4 μM each forward and reverse primer. A 1.4-kb genomic DNA

fragment containing box A1 was amplified from SAKR DNA using the primer pair 508-529 and 681-702 and reaction parameters of 20 s at 94°C, 90 s at 65°C, and 100 s at 68°C (5 cycles), followed by 20 s at 94°C, 90 s at 60°C, and 100 s at 68°C (25 cycles), with a final 10-min extension at 68°C. A 1.1-kb genomic stretch containing box A2 was amplified for 20 s at 94°C and 90 s at 68°C (25 cycles), followed by a 10-min extension at 68°C, using primer pairs spanning 980-1003 and 1111-1134. A 0.6-kb genomic fragment containing box B1 was amplified with the primer pair 899-922 and 1158-1180 for 30 s at 94°C, 90 s at 61°C, and 30 s at 68°C (5 cycles), followed by 30 s at 94°C, 90 s at 58°C, and 30 s at 68°C (25 cycles), with a final 10-min extension at 68°C. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN), followed by ethanol precipitation. Automated sequencing was performed using primers flanking the putative splice junctions. To confirm the results of this analysis and clarify any sequence ambiguities, the PCR amplicons were additionally cloned with the TOPO TA Cloning Kit (Invitrogen) to allow for sequencing of plasmid DNA.

Polymerase chain reaction analysis of isoform distribution. The RNeasy Kit (QIAGEN) was used to extract high quality RNA from fully viable cultures of SAKR, BW, PHA^{R}2.7 and CTL clone AB.1. Total cellular RNA was reverse transcribed by Superscript II using oligo(dT) or random oligonucleotide primers. Following the RT reaction, RNA was digested by incubation with 2 U of RNase H (Promega). cDNA derived from 475 ng of total RNA was analyzed for GII_{α} and GII_{β} isoform usage by PCR in 50-µl reactions employing buffer conditions and primer pairs identical to those described above for the genomic amplifications. For amplification of boxes A1 and A2,

the cycling parameters were set to 30 s at 94°C and 120 s at 68°C (5 cycles), followed by 30 s at 94°C, 90 s at 64°C, and 30 s at 68°C (5 cycles), then 30 s at 94°C, 90 s at 60°C, and 30 s at 68°C (20 cycles), with a final 10-min extension at 68°C. Box B1 was amplified under similar conditions, but using annealing temperatures of 58°C, 56°C, and 54°C. PCR reaction products were visualized by ethidium bromide staining of gels containing 2% MetaPhor agarose (FMC BioProducts) or 1.5% Ultrapure agarose (Life Technologies).

Pull-down of GII$_\alpha$ and measurement of enzymatic activity. Cells were lysed at a density of 5 x 10^7/ml in 0.5% Nonidet P-40, 1 mM Pefabloc SC, and PBS. In some experiments (as indicated) 20 mM iodoacetamide was included in the lysis buffer to inhibit formation of new disulfide linkages. Either 20 μg of GST-fusion protein or 5 μl of antiserum was added to 1 ml of post-nuclear lysate. Following a 30-min incubation on ice, fusion proteins were captured by addition of glutathione-agarose while antibodies were captured by addition of Protein A beads. In some experiments, Sepharose-4B-I3/2 and -M1/42 were also employed in the pull-down assays. Samples were placed on a rotator at 4°C for 1-2 h, after which beads were washed 3 times with 1 ml of lysis buffer. Beads were then incubated on an orbital rotator for 15 h at room temperature with 125 μl of a reaction mix consisting of 5 mM p-nitrophenyl α-D-glucopyranoside (Sigma) in PBS. As a positive control, 2.5 μl to 25 μl of the original cell lysates were incubated with the same reaction mix. For measurement of lysosomal α-glucosidase activity, the ability to hydrolyze the same substrate was tested in a reaction buffer of 100 mM sodium acetate, pH 4.5. Color change was quantitated by transferring 100 μl from each tube to a

96-well plate and measuring absorbance at 405 nm. Background absorbance, defined as the average OD_{405} value obtained when pull-downs were carried out in lysis buffer alone, was subtracted from all values obtained. In all experiments, assays were performed independently in triplicate and mean values were analyzed graphically with error bars signifying standard error of the mean.

CHAPTER III

IMMOBILIZED ANTIBODIES TO CD45 INDUCE RAPID MORPHOLOGIC CHANGES AND INCREASED TYROSINE PHOSPHORYLATION OF $p56^{lck}$-ASSOCIATED PROTEINS IN T CELLS

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A. Introduction

Although a specific ligand for CD45 has not yet been identified, the development-specific and activation-specific manner in which isoforms are expressed (Trowbridge and Thomas, 1994) may be important in regulating interactions with specific cognates on either the same cell surface or on apposing surfaces. These interactions may impinge on leukocyte activation or may mediate other novel functions. Recent studies employing chimeric proteins indicate that the PTP activity of the cytoplasmic domain is both necessary and sufficient for proximal activation responses such as tyrosine phosphorylation and $[Ca^{2+}]_i$ flux in Ab-stimulated T cells (Desai et al., 1993; Hovis et al., 1993; Volarevic et al., 1993; Desai et al., 1994). Studies have also been undertaken to evaluate the ability of the ectodomain of CD45 to modulate T-cell responses.

Transfection of a $CD45^-$, TCR^+ T lymphoma with different CD45 isoforms revealed that the no exon isoform of CD45 ($CD45^{Null}$) imparts enhanced sensitivity to Ag stimulation (Novak et al., 1994), suggesting that the external domain can influence functional outcomes. A reciprocal result was attained in thymocytes of mice transgenic for $CD45^{ABC}$; expression of this high molecular weight isoform enhanced both proximal signaling events and cell proliferation in comparison to mice transgenic for $CD45^{Null}$ (Chui et al., 1994).

Numerous groups have taken advantage of the wide range of mAbs to the CD45 extracellular domain to explore the outcomes of CD45 ligation. It has been observed that primary $CD4^+$ but not $CD8^+$ T cells pretreated with certain anti-CD45 mAbs become unresponsive to anti-TCR crosslinking (Maroun and Julius, 1994). Soluble crosslinking of CD45 prior to anti-TCR triggering has also been shown be inhibit Ca^{2+} release from internal stores, a process provocatively correlated to the redistribution of internal pools of CD45 (Shivnan et al., 1996). A mAb to CD45 has been described that inhibits IL-2 secretion, Ca^{2+} mobilization, and tyrosine phosphorylation in response to stimulation through CD3 (Goldman et al., 1992). Other mAbs to CD45 have been observed to selectively increase the proliferative response of a $CD4^+$ Th2-type clone (Wolff and Janeway, 1994). In natural killer cells, crosslinking of CD45 enhances IFN-γ production (Shen et al., 1995). In addition, it has been found that treatment of certain lymphocytes with mAbs to CD45 induces homotypic aggregation via both LFA-1-dependent and -independent pathways, depending on the mAb used (Lorenz et al., 1993; Bernard et al., 1994; Spertini et al., 1994; Zapata et al., 1995). Interestingly, T cells at different developmental stages, which by definition express different CD45 isoforms, exhibit

varying capacities to mediate homotypic adhesion in response to CD45 crosslinking (Bernard et al., 1994).

This study aims to further elucidate the role of CD45 in leukocyte activation by examining the biochemical and functional consequences of CD45 engagement by immobilized mAbs, a condition that may lead to outcomes distinct from the previous studies.

B. Results

T cells undergo rapid morphologic changes in response to immobilized anti-CD45 antibodies. Immobilized mAbs to CD45 were found to induce dramatic morphologic changes in T cells. Normally, CTL clone 11 and T-helper hybridoma A1.1 exhibit irregular spherical morphologies when grown in culture or when plated on BSA-coated microtiter wells (Fig. 3-1). However, after a 1-h incubation in wells coated with I3/2, a mAb that recognizes a pan-specific determinant of the CD45 ectodomain, the majority of these cells displayed a dramatically altered phenotype characterized by extreme cell flattening and gross irregularities in cell shape, often accompanied by the appearance of spindle-like projections (Fig. 3-1). Perhaps the most striking feature of these flattened cells, when observed by light microscopy, was their loss of refractility; the cells acquired a ghost-like appearance and almost disappeared from view.

To ascertain whether this phenomenon was specific to the anti-CD45 mAb used or whether mAbs to other highly expressed cell-surface proteins could induce similar effects, the morphologies of clone 11 and A1.1 cells were examined in the presence of an

immobilized isotype-matched mAb to MHC class I molecules, M1/42. MHC class I molecules are constitutively expressed on most cells at approximately 100,000 to 200,000 molecules/cell, levels comparable to CD45 surface expression (Barclay et al., 1993). It was observed that neither clone 11 nor A1.1 cells exhibited flattened morphologies following plating on immobilized M1/42 (Fig. 3-1). A second control mAb to LFA-1, another highly expressed surface marker, yielded similar results (Fig. 3-1).

The kinetics of this intriguing CD45-induced morphologic change were examined. Clone 11 and A1.1 cells were added to wells containing immobilized I3/2 or M1/42, and the percentage of cells exhibiting a flattened morphology was assessed at various time intervals. Data from representative experiments for each of these cell lines are presented in Fig. 3-2. On an anti-MHC class I-coated surface, neither of the cell lines exhibited flattening at any time during the 1-h incubation. However, on anti-CD45-coated wells, clone 11 and A1.1 cells exhibited rapid morphologic changes that reached peak levels within 1 h. These results also demonstrate that I3/2-induced changes are not dependent on the presence of serum components, since the incubations were carried out in D-PBS.

To extend these results, an enlarged panel of T-cell lines was assayed for mAb-induced morphologic changes. Flow cytometric analysis was conducted in parallel to establish relative levels of surface expression of CD45, MHC class I, and LFA-1 among the various cells. The results are summarized in Table 3-1. In all CD45-expressing cells surveyed, immobilized I3/2 induced dramatic morphologic changes in the majority of the cell population by 1 h. A second anti-CD45 mAb, M1/9, yielded similar results. Although different cell lines exhibited slight differences in their flattened appearance, all demonstrated the striking loss of refractility that is a defining feature of CD45-induced

morphologic alterations. Consistent with the earlier observations in A1.1 and clone 11, mAb to class I molecules and LFA-1 failed to induce the flattened morphology, even in cells expressing the highest levels of these markers. In summary, two different mAbs to CD45 induced rapid morphologic changes in T cells while two mAbs to other highly expressed surface markers failed to induce these changes. Collectively, these results suggest that the striking morphologic outcome results from specifically engaging CD45 on an immobilized surface and is not simply a consequence of trapping highly expressed cell-surface molecules.

Inhibitors of actin polymerization inhibit CD45-induced morphologic changes. The dramatic nature of the CD45-induced morphologic changes suggested that a reorganization of the actin cytoskeleton might have been triggered. To investigate whether actin polymerization is required for induction of the altered phenotype, clone 11 cells were pretreated for 15 min with various concentrations of cytochalasin (cyt) D or cyt E before addition to I3/2-coated wells. The results of a representative experiment are presented in Fig. 3-3. At inhibitor concentrations greater than 0.1 μM, the ability of cells to undergo morphologic changes is diminished. Cyt E was more potent than cyt D at preventing cell flattening; full inhibition was achieved at 0.5 μM cyt E and 5 μM cyt D. These data point to an essential role for actin polymerization in morphologic changes triggered by immobilized anti-CD45.

CD45-induced morphologic changes are inhibited by herbimycin A. Since CD45 has been implicated as an upstream regulator of various Src family PTKs during

Ag-triggered activation of lymphocytes (Trowbridge and Thomas, 1994), and PTK activation has been linked to cytoskeletal changes (Burridge et al., 1992; Fox et al., 1993), it was of interest to investigate whether CD45-induced morphologic changes are sensitive to herbimycin (herb) A, a PTK inhibitor that has been shown to reverse the abnormal morphologies of cells transformed with oncogenic PTKs (Uehara et al., 1988). Cells were preincubated with different concentrations of herb A for 15 min and the percentage of cells exhibiting altered morphologies on I3/2-coated wells after 1 h in the continued presence of the inhibitor was determined. Results from two cell lines are diagrammed in Fig. 3-4. As herb A was added at increasing concentrations (up to 10 μg/ml), cell flattening was inhibited in a dose-dependent manner. Cell viability was not diminished by these treatments as assessed by trypan blue exclusion. Thus, tyrosine kinase activity may be required for CD45-mediated morphologic events.

Immobilized anti-CD45 induces tyrosine phosphorylation events. The results of the herb A experiments suggested that specific tyrosine phosphorylation events are associated with anti-CD45-triggered morphologic changes. To investigate this issue, cells were lysed in reducing SDS sample buffer at various times following addition to mAb-coated microtiter wells, proteins were resolved by SDS-PAGE, and tyrosine-phosphorylated species were detected by immunoblotting. As shown in Fig. 3-5*A*, a rapid and sustained increased in phosphotyrosine content of a number of proteins was observed after addition of clone 11 cells to immobilized I3/2. On most Western blots, tyrosine phosphorylation of proteins of 60-80 kDa and 110-130 kDa were induced by immobilized I3/2. Although the 110- to 130-kDa species exhibited high basal levels of

phosphorylation, their phosphorylation was clearly increased upon I3/2 binding. The relative prominence of the 60- to 80-kDa phosphoproteins compared to the higher molecular weight species was found vary somewhat from experiment to experiment, and on some gels these phosphoproteins could be clearly resolved into multiple migrating species (Fig. 3-5*B*). Whether these represent distinct proteins or related phosphoisomers is not known. The increase in tyrosine phosphorylation is unlikely to be Fc-receptor meditated, as it was not observed with the control anti-transferrin receptor mAb R17 (Fig. 3-5*B*). Interestingly, a tyrosine phosphorylation response of lesser magnitude and duration than that that seen in I3/2-triggered cells was elicited when cells were plated on M1/42 (Fig. 3-5*C*). This is consistent with previous reports that Ab ligation of class I proteins induces protein tyrosine phosphorylation of multiple proteins (Bregenholt et al., 1996), an effect that is dependent on CD45 expression (Skov et al., 1995). As a control, it was confirmed that blots reacted with anti-phosphotyrosine serum revealed a similar pattern of bands to those observed when blotting was carried out using the mAb PY72 (Fig. 3-5*D*). Fig. 3-5*D* also confirms that the changes observed are not unique to CTL clone 11. Surprisingly, crosslinking of soluble CD45 mAb failed to trigger detectable tyrosine phosphorylation (data not shown). Collectively, these results demonstrate that immobilized CD45 mAb stimulates PTK activity in T cells.

Inhibitor treatments that block the changes in morphology also block tyrosine phosphorylation. Given the close temporal correlation between changes in cellular morphology and changes in tyrosine phosphorylation, it was important to examine the ability of herb A to inhibit tyrosine phosphorylation events induced by I3/2. Clone 11

cells pretreated with 0.5 to 1 μg/ml of herb A displayed a tyrosine phosphorylation profile identical to untreated cells following a 15-min incubation on immobilized I3/2 (Fig. 3-6*A*). In contrast, cells pretreated with 5 μg/ml of herb A showed some reduction in tyrosine phosphorylation, while cells treated with 10 μg/ml displayed lower levels of phosphorylation than mock-treated cells. When a kinetic analysis was carried out with cells pretreated for 15 minutes with 10 μg/ml of herb A, very slight induction of tyrosine phosphorylation was detectable by 40 minutes (Fig. 3-6*B*). Intriguingly, the extent of phosphorylation inhibition obtained in these experiments directly correlated with the degree of inhibition of morphologic changes (Fig. 3-4).

To determine whether the induction of tyrosine phosphorylation was dependent on cytoskeletal integrity, phosphorylation responses were evaluated in the presence of the cytoskeletal inhibitors. Clone 11 cells were preincubated for 15 min with concentrations of cyt D or cyt E in the inhibitory range for I3/2-induced morphologic changes. A strong reduction in levels of tyrosine phosphorylation normally induced during a 15-min incubation in I3/2-coated wells was observed at concentrations of cyt D and cyt E that inhibited cell flattening (Fig. 3-7, compare with Fig. 3-3). Interestingly, phosphorylation of the 130-kDa species remained elevated above basal levels even at high inhibitor concentrations, suggesting that some signaling can occur in the absence of actin polymerization. Overall, these observations are consistent with a requirement for increased tyrosine phosphorylation for induction of the altered morphology.

Comparison of anti-CD45 and anti-CD3-induced phosphorylation. Antibodies to the TCR complex are known to induce the tyrosine phosphorylation of multiple

proteins (Chan et al., 1994). In addition, immobilized mAbs to CD3 have been shown to induce reorganization of the actin cytoskeleton in a human T-cell line (Parsey and Lewis, 1993). Since the present data suggested that anti-CD45-triggered cells also undergo actin reorganization and tyrosine phosphorylation, a comparison was made between the tyrosine phosphorylation profile induced by I3/2 and that induced by 2C11, a mAb that recognizes the ε subunit of the CD3 complex. As shown in Fig. 3-8, the spectrum of phosphoproteins observed in clone AB.1 cells stimulated with either of these mAbs appeared to comigrate. At longer time points, however, it is clear that high levels of anti-CD3ε lead to the phosphorylation of additional proteins not induced by anti-CD45. The induction of phosphorylation of these additional proteins may be related to unique functional responses triggered by anti-CD3ε mAb. Interestingly, when high levels of both I3/2 and 2C11 were immobilized in the same well, the kinetics of phosphorylation were increased; this was particularly apparent at the early (5 min) time point. Furthermore, coimmobilization of I3/2 with sub-stimulatory levels of anti-CD3ε (the $2C11^{lo}$ condition, as determined in Ostergaard and Ma, 1995) appeared to increase the total phosphorylation response achieved by 15 min. Although it is not clear whether this acceleration of phosphorylation kinetics was due to enhanced adhesion to the mAb-coated surface and/or true synergy of signals, these data raise the possibility that certain redundancies exist at the level of signal transduction cascades triggered by immobilized mAbs to CD3ε or CD45. It is possible that this is related to the ability of both pathways to activate actin polymerization in T cells.

Tyrosine phosphoproteins induced by immobilized anti-CD45 mAb specifically coimmunoprecipitate with p56^{lck}. Experiments undertaken to determine the identities of p60-80 and p110-130 yielded negative results. Nevertheless, the intriguing observation was made that immunoprecipitation of the Src family PTK p56^{lck} was accompanied by precipitation of 60-80-kDa and 110-130-kDa phosphoproteins that comigrate with the anti-CD45 induced phosphoproteins identified in total cell lysates (Fig. 3-9). The association between these tyrosine phosphoproteins and p56^{lck} is specific, since none of these proteins were detected in parallel immunoprecipitates using preimmune rabbit serum. The association of these proteins with p56^{lck} was preserved even after washing the immunoprecipitates with a stringent buffer containing both SDS and DOC, suggesting that these molecules exist in stable complexes with the PTK. Interestingly, p110-130, which exhibit some basal phosphorylation, were found to be capable of associating with p56^{lck} in resting cells. It is not clear whether the additional phosphoproteins observed in p56^{lck} immunoprecipitates from stimulated cells are constitutively associated with p56^{lck} and become phosphorylated after binding of cells to anti-CD45 or if these proteins become associated with p56^{lck} after becoming tyrosine phosphorylated. In summary, these data demonstrate that engagement of the CD45 external domain, in the absence of additional stimulation, is sufficient to induce changes in phosphoproteins that associate with p56^{lck}.

Contribution of p56^{lck} to anti-CD45-induced phosphorylation and morphology changes. Having established a physical association between p56^{lck} and p60-80/p110-130, it remained to be determined whether p56^{lck} plays a role in the phosphorylation of these

molecules. To address this issue, CTLL-2 cells and a $p56^{lck}$-deficient variant of this line (Karnitz et al., 1992) were compared for their phosphorylation responses to immobilized I3/2. As shown in Fig. 3-10, phosphorylation of p60-80 and p110-130 was enhanced in I3/2-stimulated CTLL-2 cells, although the response was not as striking as that observed in AB.1 cells. Significantly, induction of phosphorylation of these molecules could not be detected in $p56^{lck}$-deficient CTLL-2 cells (Fig. 3-10). It was interesting that the CTLL-2 cells did not undergo dramatic morphologic changes (data not shown), as did the AB.1 cells, which correlated with the significantly dampened phosphorylation response in the CTLL-2 cells.

Since wild-type CTLL-2 cells did not undergo morphologic changes comparable to those observed in other cell lines, the issue of the contribution of $p56^{lck}$ to this phenomenon was addressed by comparison of the human Jurkat T-cell line and a $p56^{lck}$-deficient variant, J.CAM1.6 (Straus and Weiss, 1992). Upon stimulation of Jurkat cells with an immobilized anti-human CD45 mAb, GAP 8.3, striking morphologic changes were observed consistent with those occurring in mouse T cells (Fig. 3-11). As a negative control, it was verified that these changes did not occur in CD45-deficient J45.01 cells (Koretzky et al., 1991). Significantly, $p56^{lck}$-deficient J.CAM1.6 cells also failed to undergo detectable changes in response to immobilized GAP 8.3. These data confirm that CD45-induced morphologic changes occur in the human system and illustrate the central role of $p56^{lck}$ in mediating these changes. These data do not, however, exclude the possibility that other PTKs are also involved in anti-CD45-triggered signaling cascades.

C. Summary of Results

Herein, it is demonstrated that immobilized mAbs to the external domain of CD45 induce rapid and dramatic morphologic changes in a variety of T-cell lines, including $CD8^+$ CTL clones. CD45-induced morphologic changes are blocked by the cytoskeletal inhibitors cyt D and cyt E and by the PTK inhibitor herb A. Consistent with the requirement for PTK activity, tyrosine phosphorylation of multiple proteins is triggered by immobilized anti-CD45 mAb. Interestingly, the kinetics of phosphorylation closely parallel the kinetics of morphologic changes and the spectrum of phosphoproteins induced is similar to that observed in cells plated on immobilized anti-CD3ε. Furthermore, the induction of tyrosine phosphorylation is inhibited by PTK and cytoskeletal inhibitors at drug concentrations that also inhibit the changes in morphology. The phosphoproteins induced by immobilized anti-CD45 stimulation are coimmunoprecipitated with $p56^{lck}$, suggesting that this PTK might play a role in their phosphorylation. Consistent with this, tyrosine phosphorylation events are not triggered in $p56^{lck}$-deficient CTLL-2 cells. An important role for $p56^{lck}$ in the morphologic pathway is further supported by the observation that $p56^{lck}$-deficient human J.CAM1.6 cells, in contrast to the parental Jurkat line, cannot be induced to undergo morphologic changes.

D. Discussion

Coordination between tyrosine phosphorylation and cytoskeletal rearrangement. There now exists a significant body of literature revealing an intimate link between tyrosine phosphorylation and cytoskeletal reorganization. The focal adhesion kinase, $p125^{FAK}$, is a cytosolic PTK that localizes to sites of cell-to-matrix attachment, termed focal adhesions, that are rich in actin stress fibers (Burridge et al., 1997). Recently, $p125^{FAK}$ has been shown to physically associate with the cytoskeletal proteins talin (Chen et al., 1995) and paxillin (Hildebrand et al., 1995; Salgia et al., 1996). In $p125^{FAK}$-deficient cells, the number of focal adhesions is increased and cell motility is diminished, indicating that this PTK may function in focal adhesion turnover occurring during cell migration (Ilic et al., 1995; Ilic et al., 1996). In neurons, the phosphorylation of $p125^{FAK}$ is dependent upon intact F-actin, and can be abolished by cyt D (Zhang et al., 1996). Activation of $p125^{FAK}$ by integrin-matrix interactions is associated with the phosphorylation of paxillin on tyrosine, a process inhibitable by herb A (Burridge et al., 1992), a drug which also reduces fibroblast spreading on extracellular matrix (Uehara et al., 1988). Interestingly, LAR, a PTP related to CD45 that is expressed in fibroblasts, localizes to focal adhesions and sites of F-actin disassembly (Serra-Pages et al., 1995). Members of the Src family of PTKs have also been identified as potential mediators of cytoskeletal changes. Paxillin is known to be a substrate for Src family kinases and there are physical interactions between members of this PTK family and $p125^{FAK}$ (Cobb et al., 1994). In addition, cells transformed with $p60^{src}$ display morphologic abnormalities accompanied by elevated tyrosine phosphorylation of multiple

cytoskeletal components, including paxillin, tensin, talin, and vinculin (Schaller and Parsons, 1993).

The data obtained in the present study suggest the existence of a positive feedback loop between protein tyrosine phosphorylation and cytoskeletal reorganization. The physical interaction of CD45 with fodrin (Lokeshwar and Bourguignon, 1992) provides one explanation for how ligation of this PTP may trigger localized changes in cytoskeletal architecture. Subtle alterations in the cytoskeletal matrix may impinge on the interactions of signal transducing molecules, setting into motion a cascade of events that accelerate cytoskeletal changes and, in concert, augment protein tyrosine phosphorylation. Indeed, an increasingly popular notion is that signal transduction cascades are not purely chemical in nature but are subject to regulation by mechanical forces (Forgacs, 1995; Liu et al., 1996; Chen et al., 1997; Maniotis et al., 1997). In fibrobasts, for example, the ability of the adapter protein Shc to associate with F-actin is blocked by cyt D (Thomas et al., 1995). In the present study, the ability of cyt D and cyt E to inhibit tyrosine phosphorylation indicates that the accumulation of cellular phosphotyrosine is dependent upon mechanical/cytoskeletal events associated with PTK activation. Conversely, the ability of $p56^{lck}$ deficiency or herb A treatment to block morphologic changes highlights the role of tyrosine phosphorylation events in transmitting signals required for cytoskeletal redistribution. The fact that this positive feedback loop requires ligation of CD45 on an immobilized surface highlights the essential role of matrix rigidity in strengthening cytoskeletal linkages, a phenomenon that has been documented (Choquet et al., 1997).

Role of p56^{lck} in CD45-triggered events. Given the involvement of PTK mediators in cytoskeletal events, it is intriguing that p60-80 and p110-130 are found to stably interact with p56^{lck}. Further, the results with the p56^{lck}-deficient CTLL-2 cells reveal that p56^{lck} is required for detectable enhancement of phosphorylation of these proteins in response to immobilized anti-CD45 mAb, while the results obtained with the J.CAM1.6 mutant reveal a critical role for this PTK in facilitating actin-dependent changes in morphology. The relative contributions of the SH2, SH3, and kinase domains of p56^{lck} to these phenomena are not yet known, nor is whether in certain cell types other Src family kinases can partially substitute for these functions. Interestingly, p59^{fyn} and p60^{src} have been found to perform both unique and redundant functions in coordinating cytoskeletal changes in other systems (Thomas et al., 1995).

Identification of phosphoproteins induced during CD45 ligation. Recently, three of the proteins that become tyrosine phosphorylated during either anti-CD45 or anti-CD3 triggering have been identified as paxillin, p125^{FAK}, and the p125^{FAK}-related PTK Pyk2 (Ostergaard et al., 1998). All three of these proteins are capable of associating directly and in a phosphotyrosine-dependent manner with the SH2 domain of p56^{lck} (Berg and Ostergaard, 1997; Ostergaard et al., 1998). Consistent with the results of the present study, the phosphorylation of paxillin in anti-CD45 triggered T cells is dependent upon expression of p56^{lck} (Ostergaard et al., 1998). That paxillin is implicated in this signal transduction cascade is intriguing, because it contains multiple protein-protein interaction domains (Salgia et al., 1995) and has shown to be capable of interacting with the PTKs Pyk2, Csk, and p60^{src}, the adaptor protein Crk, and the cytoskeletal proteins talin and

vinculin (Hildebrand et al., 1995; Salgia et al., 1995; Schaller and Parsons, 1995; Salgia et al., 1996; Ostergaard et al., 1998). Thus, paxillin may function as a protein scaffold linking cytoskeletal structural proteins and signal-transducing modules following its phosphorylation by a $p56^{lck}$-dependent pathway. Tyrosine phosphorylation of paxillin is necessary for formation of a stable, SH2-dependent interaction with $p56^{lck}$ (Ostergaard et al., 1998), which may lead rapidly to further phosphorylation of paxillin and/or paxillin-associated proteins. Interestingly, the associations of Crk, Csk, and $p60^{src}$ with paxillin are also phosphotyrosine-dependent (Schaller and Parsons, 1995).

Synergy between CD45- and TCR-induced responses. The fact that the phosphoproteins induced by immobilized anti-CD45 share common identities (Ostergaard et al., 1998) and gel mobility profiles with those induced by anti-CD3 raises the possibility that CD45, perhaps in collaboration with $p56^{lck}$, is capable of activating certain TCR signaling pathways. Such an interpretation is consistent with a requirement for CD45 and Src family PTKs in TCR-mediated signaling (Chan et al., 1994) and is further supported by data physically linking CD45 to components of the TCR complex (Volarevic et al., 1990; Furukawa et al., 1994). The results of the present study, and that of a previous study employing anti-CD3 (Parsey and Lewis, 1993), reveal that CD45 and CD3 functionally couple to the actin cytoskeleton, consistent with the ability of immobilized mAbs to these proteins to induce phosphorylation of proteins implicated in cytoskeletal regulation (Ostergaard et al., 1998). A critical role for the actin cytoskeleton in lymphocyte activation is indicated by the finding that the productive interaction of a T lymphocyte with an APC requires the formation of a large, dynamic contact surface, a

process inhibited by cyt D (Valitutti et al., 1995a). Cytoskeletal changes activated by CD45/CD3 may thus permit the strengthening of adhesive interactions during Ag recognition, promoting further amplification of stimulatory signals.

In addition to the possible ability of CD45 to potentiate cellular activation on a physical/structural level, it is possible that the ability of CD45 ligation to activate a subset of TCR signals may function at a chemical level to reduce the requirements for TCR occupancy (i.e. the "activation threshold"). The ability of $p56^{lck}$ to couple to T-cell costimulatory pathways (Rabb et al., 1995) may also be relevant in this context. Interestingly, engagement of CD45 by the lectin-like B cell-surface protein CD22 has been found to enhance TCR-triggered tyrosine phosphorylation events (Sgroi et al., 1995), although this result must be interpreted with caution since CD22 may bind additional glycoproteins (Trowbridge and Thomas, 1994).

Novel CD45 functions. Since a specific ligand for CD45 has not yet been identified, the use of immobilized mAbs provides an *in vitro* system for studying the potential outcomes of CD45 ligation *in vivo*. As discussed, engagement of CD45 may potentiate Ag-specific signaling cascades at a chemical level by providing costimulatory signals and/or at a mechanical level by facilitating cytoskeletal changes associated with conjugate formation. The ability of a specific ligand to trigger dynamic changes in the actin cytoskeleton independent of Ag receptor engagement might also influence adhesive or migratory events occurring outside the context of Ag stimulation. Ligation of CD45 may induce responses by direct effects on the enzymatic activity of the cytoplasmic domain, as has been suggested by studies involving a chimera composed of the CD45

cytoplasmic domain attached to the extracellular domain of EGFR (Desai et al., 1993; Majeti et al., 1998). Alternatively, ligation of CD45 may induce or disrupt associations with proteins that physically interact with CD45. Further work clarifying these mechanisms may shed new insights into novel biological functions of CD45.

TABLE 3-1

Comparison of profiles of surface marker expression to sensitivity to mAb-induced morphologic changes in a panel of T-cell lines

	M1/42		M17/5.2		I3/2		M1/9	
Cell line	**MF[a]**	**MC[b]**	**MF**	**MC**	**MF**	**MC**	**MF**	**MC**
Clone AB.1	+++	–	++++	–	++	++++	++	++++
Clone 10/1	+++	–	+++	–	++	+++	ND	ND
Clone 11	++++	–	+++	–	+	+++	+	+++
A1.1	+	–	+	–	++	+++	++	+++
AODH7.1	–	–	++	+	++++	+++	++++	+++
EL4	+	–	+	+	+++	++++	+++	+++
NS-1	–	–	–	–	–	–	ND	ND

[a] Mean channel fluorescence intensity readings from at least two independent experiments were averaged. The highest average mean channel fluorescence score for each mAb condition was assigned a value of 100% and all other readings were normalized to this. Data are grouped according to percentage of maximal mean channel fluorescence for each mAb as follows: (–) 0-10%; (+) 10-30%; (+ +) 30-60%; (+ + +) 60-90%; (+ + + +) 90-100%. ND = not determined.

[b] Percent morphologic change was assessed after 1 h. Data are expressed as follows and are representative of at least three independent experiments: (–) 0-10%; (+) 10-30%; (+ +) 30-60%; (+ + +) 60-90%; (+ + + +) 90-100%. ND = not determined.

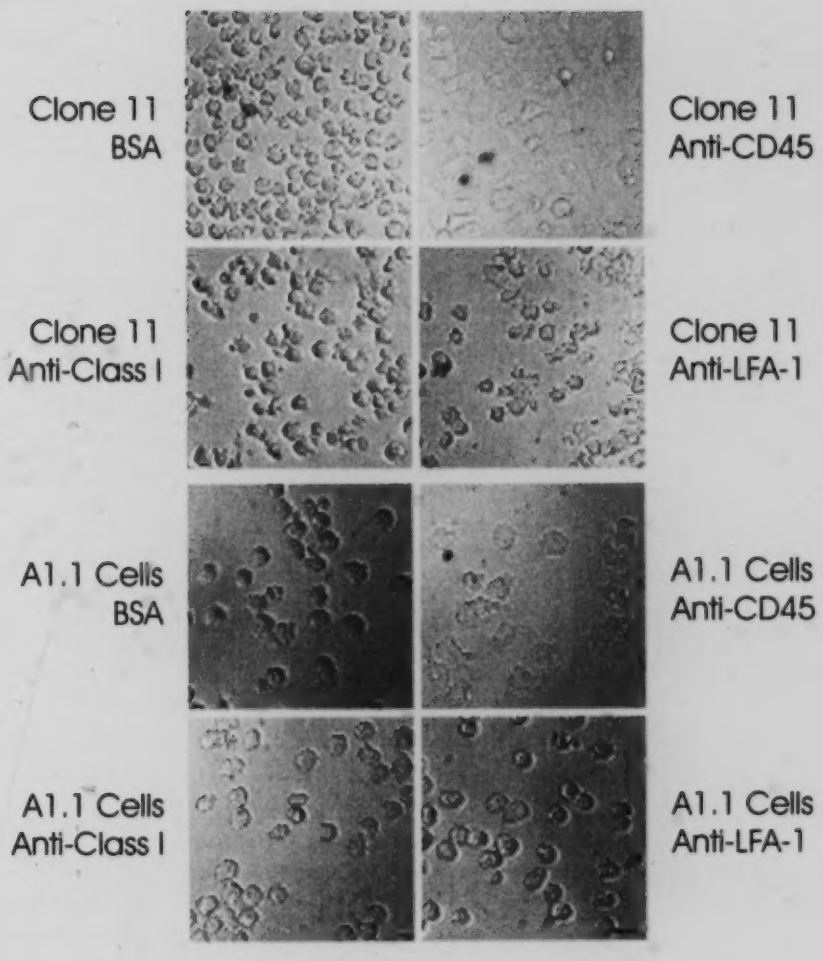

FIGURE 3-1. Effects of immobilized antibodies on T-cell morphology. Shown are light photomicrographs of CTL clone 11 or T-hybridoma A1.1 cells incubated for 1 h at 37°C in microtiter wells containing immobilized anti-CD45 (I3/2), anti-class I MHC (M1/42), anti-LFA-1 (M17/5.2) or BSA blocking protein. Bar = 10 μm.

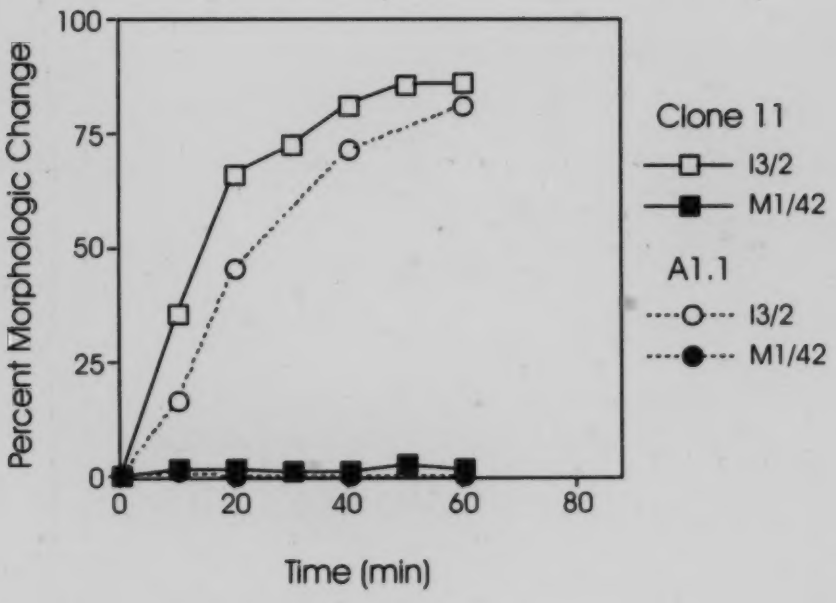

FIGURE 3-2. Kinetics of antibody-induced morphologic changes. The percentages of A1.1 and clone 11 cells exhibiting changes in morphology at various times on anti-CD45 (*I3/2*) or anti-class I MHC (*M1/42*) are indicated. Cells were added to microtiter wells in 100 µl D-PBS at 0 min and allowed to settle. Cell counts from duplicate wells were averaged, and data from one of three representative experiments are shown.

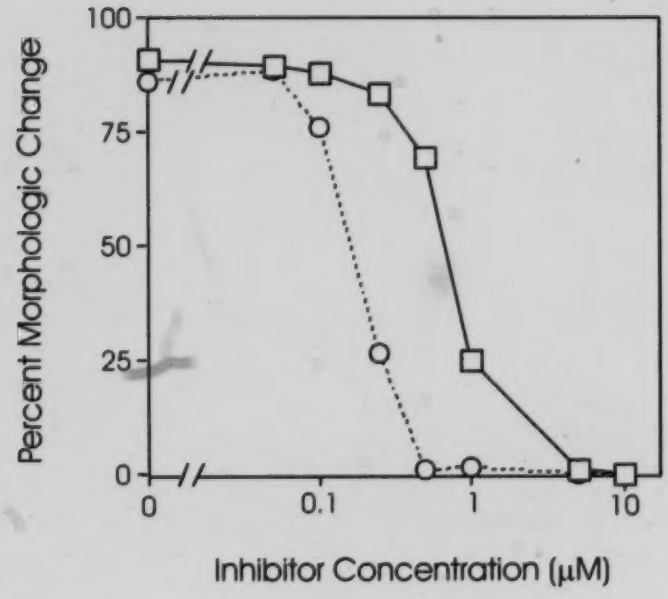

FIGURE 3-3. Effects of cytochalasin D and cytochalasin E on CD45-induced morphologic changes. Clone 11 cells were preincubated for 15 min with various concentrations of cyt D (*squares*) or cyt E (*circles*) and then added to microtiter wells containing immobilized I3/2. The percentage of cells exhibiting changes in morphology after 1 h was calculated from duplicate wells. Data plotted are from one of three representative experiments. Vehicle alone had no visible effect on the cells.

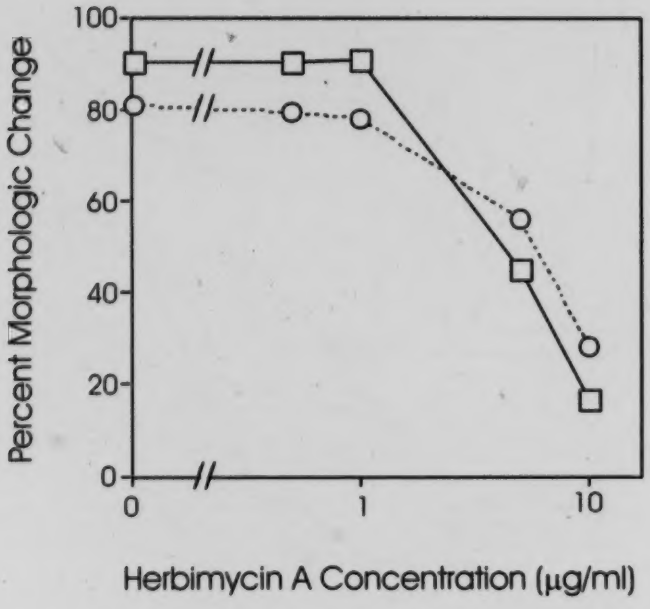

FIGURE 3-4. Effect of herbimycin A on CD45-induced changes in morphology. Clone 11 cells (*squares*) or clone AB.1 cells (*circles*) were preincubated with various concentrations of herb A before plating onto I3/2-coated wells. The percent morphologic change was assessed at 1 h and is representative of three experiments.

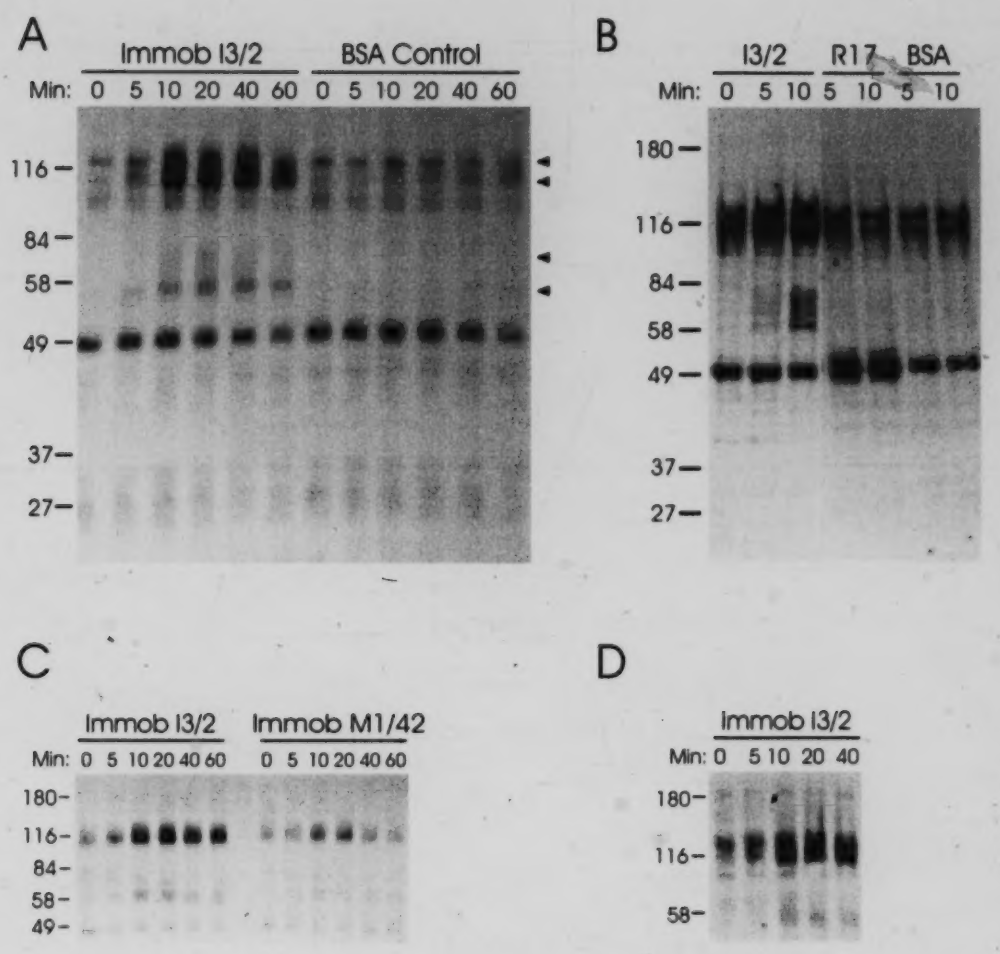

FIGURE 3-5. Kinetics of antibody-induced changes in tyrosine phosphorylation. Cells were added to coated microtiter wells at time zero and lysed after various incubation periods at 37°C. Proteins were resolved by SDS-PAGE, followed by anti-phosphotyrosine immunoblotting using mAb PY72 (*A-C*) or polyclonal antiserum (*D*). *A*, CTL clone 11 on I3/2- or BSA-coated wells. *Arrowheads* indicate regions of phosphotyrosine induction by anti-CD45. *B*, comparison of clone 11 cells on I3/2, R17 (anti-transferrin receptor), or BSA at two time points. The longer exposure time compared to *A* reveals the induction of numerous distinct phosphoproteins in the 60- to 75-kDa range after anti-CD45 stimulation. *C*, comparison of tyrosine phosphorylation events in clone 11 cells plated on I3/2 versus the class I mAb M1/42. *D*, examination of I3/2-induced phosphorylation events in CTL clone AB.1.

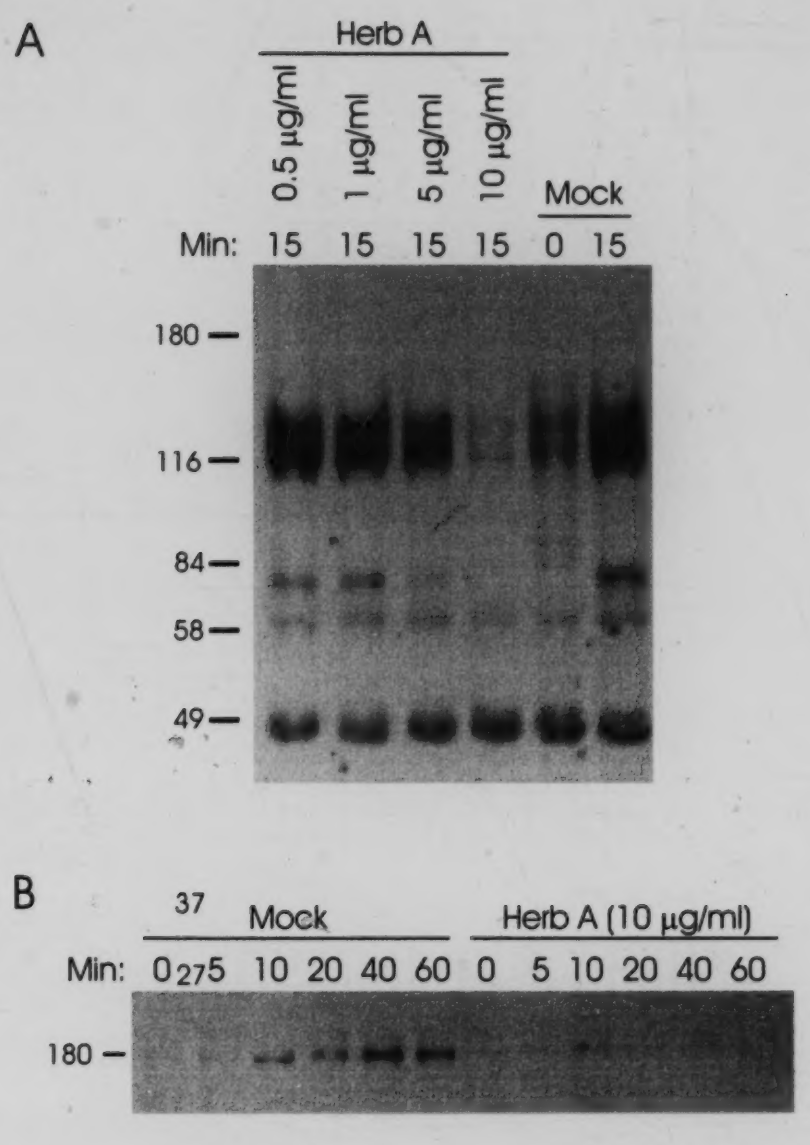

FIGURE 3-6. Effects of herbimycin A treatment on CD45-induced tyrosine phosphorylation events. *A,* shown is an anti-phosphotyrosine blot of total cell lysates from clone 11 cells preincubated for 15 min with the indicated concentrations of herb A or with DMSO vehicle alone (*mock*). Cells were lysed after 0 min or 15 min in I3/2-coated wells. *B,* clone 11 cells were pretreated for 15 min with 10 µg/ml herb A or vehicle alone (*mock*). The cells were then stimulated with immobilized I3/2 and lysates from 5 x 10^4 cells were collected after the indicated incubation periods. Tyrosine phosphorylated proteins were analyzed by Western blot.

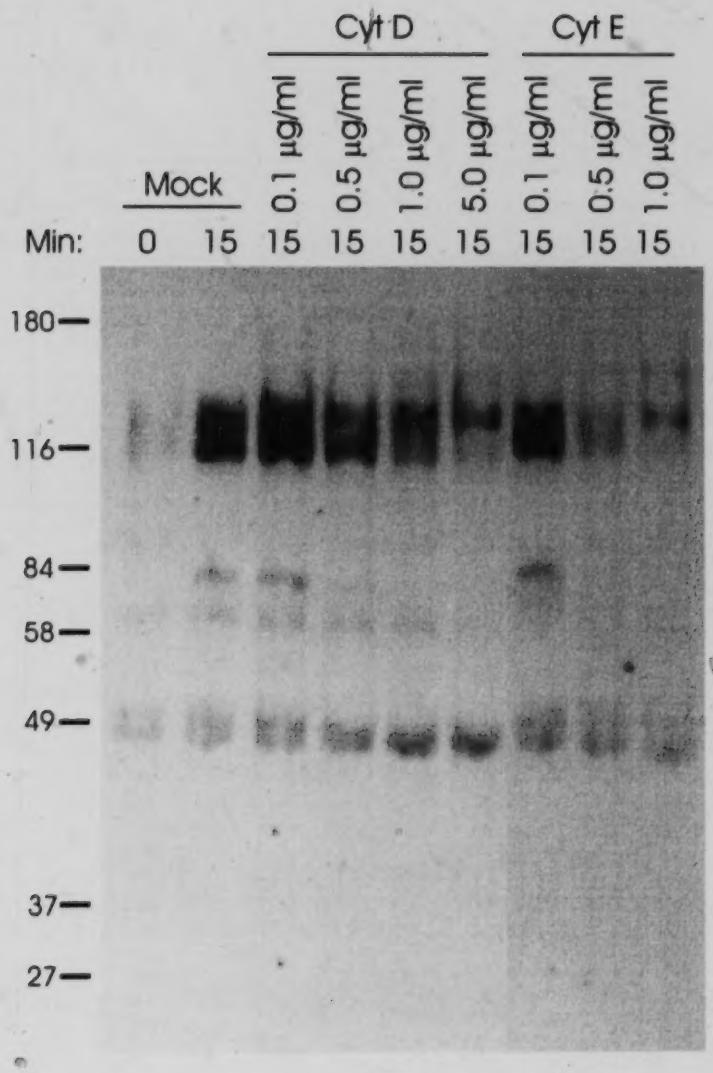

FIGURE 3-7. Dose-dependent effects of cytochalasin D and cytochalasin E on CD45-induced tyrosine phosphorylation events. Clone 11 cells were incubated for 15 min with the indicated concentrations of cyt D or cyt E before addition to I3/2-coated microtiter wells. Cells were lysed at 0 min or 15 min after plating. Mock treated cells were preincubated with ethanol carrier alone. Western blotting was carried out to examine tyrosine-phosphorylated proteins.

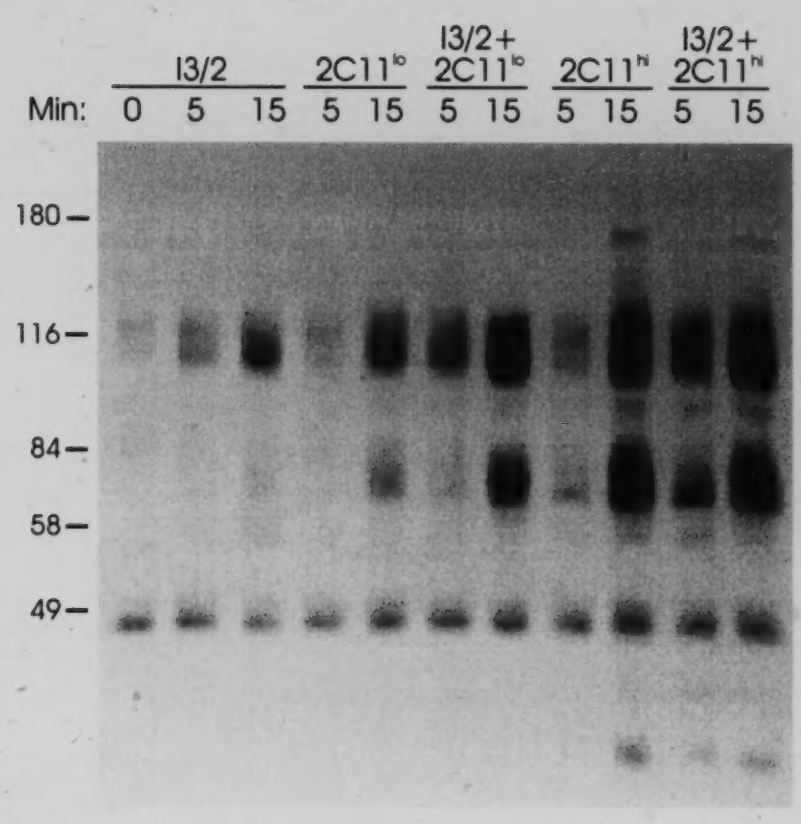

FIGURE 3-8. Comparison of anti-CD45-induced and anti-CD3-induced tyrosine phosphorylation events. Clone AB.1 cells were added to wells precoated with I3/2, sub-stimulatory levels of anti-CD3ε ($2C11^{lo}$), maximal stimulatory levels of anti-CD3ε ($2C11^{hi}$), or combinations of these. Total cell lysates were prepared after 0 min, 5 min, or 15 min at 37°C. The blot was probed with anti-phosphotyrosine Ab.

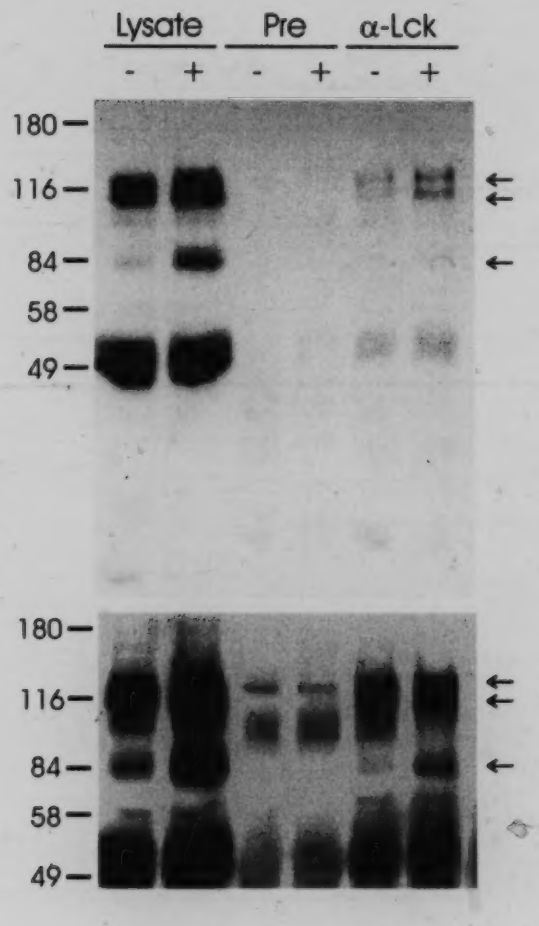

FIGURE 3-9. Association of anti-CD45-induced phosphoproteins with p56^{lck}. Clone 11 cells were incubated for 30 min on BSA (-) or I3/2 (+)-coated wells followed by lysis and immunoprecipitation with either preimmune rabbit serum (*Pre*) or anti-p56^{lck} antiserum (α-*lck*). Immunoprecipitates were washed with RIPA buffer and resolved by SDS-PAGE for identification of tyrosine phosphorylated proteins by immunoblotting. Lysates from approximately 3 x 10^6 cells were pooled for each immunoprecipitation. Lysates (*Lys*) from 2 x 10^5 cells are presented in the first two lanes. Two different exposures of the same blot are shown for comparison. *Side arrows* identify phosphoproteins induced by anti-CD45.

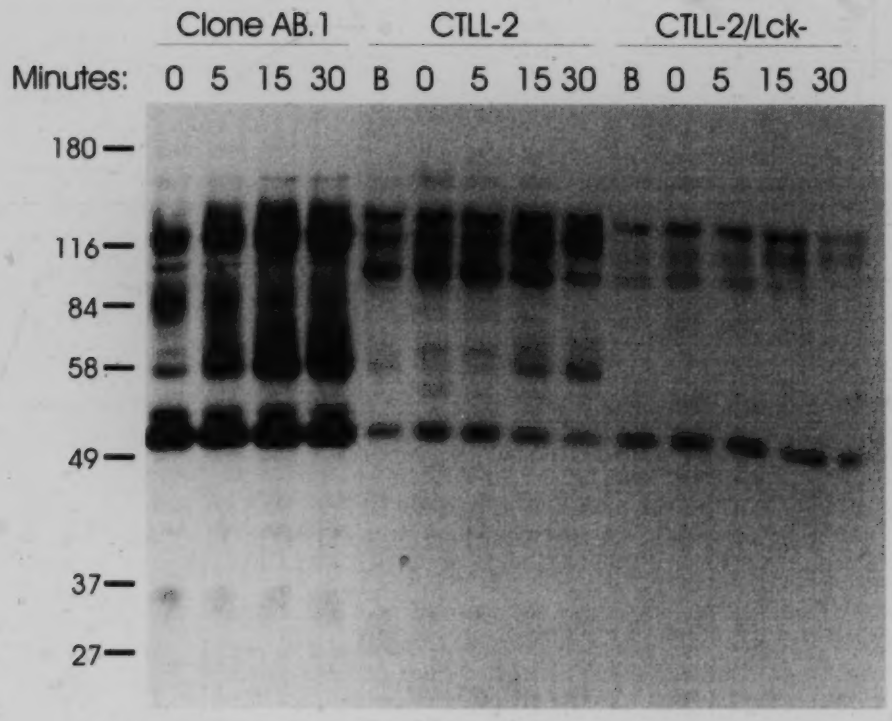

FIGURE 3-10. Role of p56^{lck} in CD45-induced phosphorylation events. AB.1, CTLL-2, or p56^{lck}-deficient CTLL-2 cells were incubated on I3/2-coated wells and lysed in reducing SDS sample buffer at 0, 5, 15, and 30 min. As a control, the CTLL-2 lines were also lysed after 30 min on BSA-coated wells (lanes marked *B*). Tyrosine phosphorylated proteins were revealed by Western blotting.

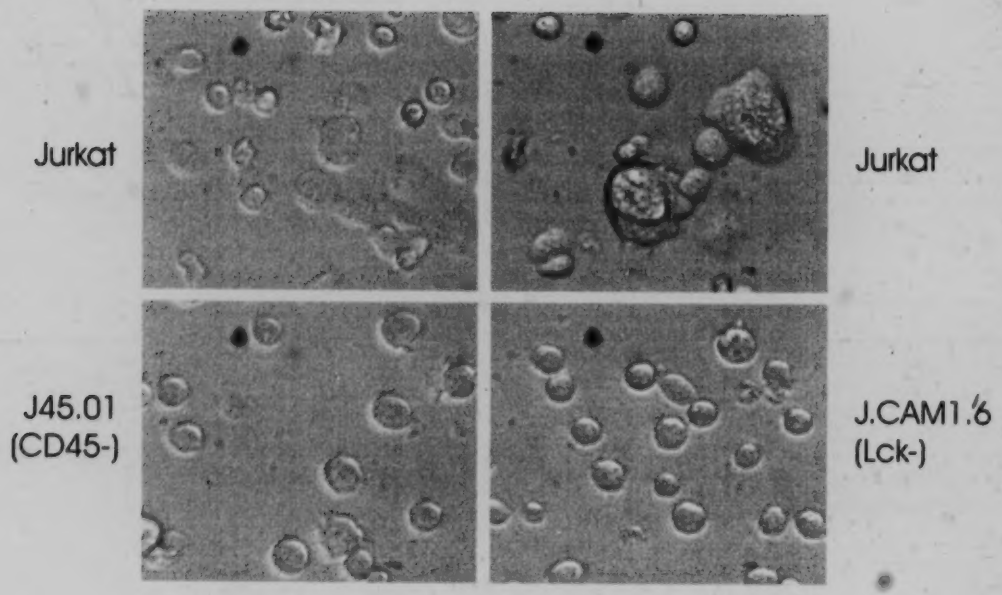

FIGURE 3-11. Jurkat deficient p56^{lck} do not undergo anti-CD45 induced morphologic changes. Jurkat, J45.01, and J.CAM1.6 cells were photographed after a 2-h incubation in wells coated with mAb GAP 8.3, recognizing human CD45.

CHAPTER IV

CD45 PROTEIN-TYROSINE PHOSPHATASE IS SPECIFICALLY ASSOCIATED WITH A 116-kDa TYROSINE-PHOSPHORYLATED GLYCOPROTEIN

(A version of this chapter has been published. Arendt, C. W. and Ostergaard, H.L. 1995. Journal of Biological Chemistry. 270(5): 2313-2319.)

A. Introduction

CD45 is a high molecular weight transmembrane PTP abundantly expressed on nucleated cells of hematopoietic origin that has been shown to function in both T- and B-lymphocyte activation (Trowbridge and Thomas, 1994). It is unclear at present how the PTP activity of CD45 is controlled and whether its regulation of Src family kinases is its only role during lymphocyte activation. Since the enzymatic activity and cellular functions of CD45 are likely to be regulated through intermolecular interactions, considerable efforts have been made to identify proteins that physically associate with CD45. As previously summarized in Chapter I, a variety of proteins have been proposed to interact with CD45 based on studies that most commonly employ techniques such as chemical crosslinking and immunofluorescent cocapping. Coimmunoprecipitation studies have also been performed under conditions of Brij or digitonin lysis using Western blot analysis or radiolabeling techniques to detect interactions between CD45 and other proteins. One difficulty in interpreting these data is that CD45 accounts for up

to 10% of the total cell-surface protein on lymphocytes (Thomas, 1989), raising the possibility that these high sensitivity assays might be capable of detecting proteins that transiently but non-specifically interact with CD45. Interestingly, proteins that are capable of being captured in detergent micelles with CD45 under conditions of Brij or digitonin lysis often do not associate with CD45 when the nonionic detergent NP-40 is employed (Schraven et al., 1990; Schraven et al., 1991; Torimoto et al., 1991; Koretzky et al., 1993; Takeda et al., 1994; Herold et al., 1996). Moreover, many of the studies carried out to date have identified CD45-associated proteins that are expressed on subsets of lymphocytes. It is unclear whether there are alternative molecules associated with CD45 in other cell types or whether there are ubiquitously expressed molecules associated with CD45 in all hematopoietic cells.

The goal of this present study is to identify molecules that remain associated with CD45 in the detergent NP-40. It is hypothesized that CD45 engages in specific protein-protein interactions that are stable in NP-40 and that occur at sufficiently high stoichiometry to be detected by total protein staining of proteins resolved in polyacrylamide gels.

B. Results

CD45 specifically associates with a 116-kDa protein in a T-lymphoma cell line. To identify proteins that specifically coimmunoprecipitate with CD45, the SAKR T lymphoma and a CD45-negative variant of this cell termed SAKR/CD45$^-$ (Hyman and Trowbridge, 1981) were solubilized in buffer containing 0.5% NP-40. CD45 was immunoprecipitated with the Sepharose 4B-coupled pan-specific mAb I3/2 and the bead

fraction was washed extensively with lysis buffer before being subjected to SDS-PAGE. To minimize non-specific interactions, the Sepharose 4B beads were blocked with 3% BSA prior to use for immunoprecipitation. Proteins precipitated in the immune complexes were initially resolved on polyacrylamide gels for visualization by silver staining (Fig. 4-1*A*). In addition, separated proteins were transferred to Immobilon-P for staining with India ink (Fig. 4-1*B*). Under both detection conditions, a protein with an estimated molecular mass of 116 kDa was observed to coimmunoprecipitate with CD45. The 116-kDa protein (p116) was not, however, precipitated from SARK/CD45$^-$ cells. When immunoprecipitates were prepared using M1/42, a mAb of the same isotype as I3/2 but recognizing MHC class I molecules (which are ubiquitously expressed), no protein of identical mobility to p116 was obtained (Fig. 4-1*B*). Coprecipitation of p116 with CD45 was not restricted to the I3/2 reagent, but also occurred when another pan-specific anti-CD45 mAb, M1/9, was employed (Fig. 4-1*B*). An antiserum directed against the entire cytoplasmic domain of CD45 was unable to react with p116 by Western blot assay, suggesting that p116 is not a proteolytic breakdown product of CD45 (Fig. 4-1*C*). The CD45-associated protein was found to be present in immunoprecipitates of SAKR but not SAKR/CD45$^-$ cells following metabolic labeling with [^{35}S]methionine (Fig. 4-1*D*). No change in the association was observed if cells were treated with the Ca^{2+} ionophore ionomycin as an activation stimulus prior to lysis (Fig. 4-1*D*). Taken together, these results indicate that the 116-kDa protein specifically associates with CD45 as opposed to non-specifically associating with the immunoprecipitating Ab or bead matrix. The association is not due to Ab cross-reactivity because p116 can be immunoprecipitated by two independent anti-CD45 mAbs and is not immunoprecipitated from lysates of CD45$^-$ cells.

p116 is associated with CD45 in multiple cell types. Cell lines derived from a number of different hematopoietic lineages were screened to determine whether p116 is associated with CD45 in all cells that express CD45. Since CD45-negative variants are not available for many of these cell lines, I3/2 immunoprecipitates were compared to parallel immunoprecipitates employing isotype-matched M1/42 as a negative control. A sampling of results from these experiments is presented in Fig. 4-2. By inspection of India ink stained membranes, a 116-kDa protein was clearly observed to be present in I3/2 but not M1/42 immunoprecipitates from CTL clone 2C, AKR thymoma, and LK35 B-hybridoma cells (Fig. 4-2*A*). A 116-kDa protein was also found to co-immunoprecipitate with CD45 from the T-lymphoma EL4, an association that was not altered by pretreating the cells with an activating concentration of the phorbol ester TPA (Fig. 4-2*B*). Interestingly, a 116-kDa protein was found to associate with CD45 but not class I MHC molecules from additional B- and T-cell tumor lines (A20.CY, NZB.1, BW, YAC-1), T-cell hybridomas (AODH 7.1, A1.1), and the mastocytoma P815 (data not shown). Therefore, p116 is expressed in a variety of cell lineages and is capable of associating with CD45 in these cells.

p116 associates with the external domain, transmembrane domain, and/or membrane-proximal region of CD45. A revertant of the BW/CD45$^-$ thymic lymphoma line that reexpresses CD45 has been isolated. The CD45 expressed by BW/rev, however, is truncated such that most of the cytoplasmic domain is deleted, as demonstrated by *in vivo* inorganic phosphate labeling studies (Hyman et al., 1982). Although the precise residues at which the truncation occurs have not yet been mapped, the CD45 that is

expressed is approximately 120 kDa which is consistent with it lacking the entire cytoplasmic domain but possessing a fully intact external domain (Hyman et al., 1982). Interestingly, the association between CD45 and p116 was found to be intact in the BW/rev cell line (Fig. 4-3). The amount of the 116-kDa protein immunoprecipitated with CD45 from BW/rev is much lower than in the BW parent, consistent with the finding that CD45 expression is reduced 80-90% in the mutant cell (Ostergaard et al., 1989). The observation that p116 is found to associate with this truncated form of CD45 suggests that it associates with the external domain, the transmembrane domain, and/or the membrane-proximal region of CD45.

p116 is not CD45 isoform-restricted in its association. Since p116 may associate with the external domain of CD45, it is possible that the differential expression of exons 4-6 (A-C), encoding a region near the amino terminus of CD45, might impinge on the association with p116. To address this issue, mouse fibroblast ψ2 cells were obtained that had been infected with recombinant retrovirus engineered to direct the expression of various individual isoforms of CD45 (Johnson et al., 1989b). The CD45 isoforms expressed by these cells include $CD45^{ABC}$, $CD45^{BC}$, $CD45^{C}$, and $CD45^{Null}$, where the latter completely lacks the region encoded by the three alternatively spliced exons. To assess the ability of these isoforms to associate with p116, CD45 immunoprecipitates were prepared from each of these cell lines, as well as from a control-infected line. It was hypothesized that if CD45 and p116 engage in a stable and specific interaction, then by mixing the beads containing individual CD45 isoforms with [^{35}S]Met-labeled lysates from SAKR/CD45^{-} cells it might be possible to reconstitute the association *in vitro*. Using CD45 immunoprecipitated from SAKR cells as a positive control, it was found that

in vitro reconstitution of the association was indeed possible (Fig. 4-4*A*). Interestingly, each of the four different CD45 isoforms immunoprecipitated from the ψ2 cells appeared to associate equally well with a metabolically labeled 116-kDa protein from SAKR/CD45$^-$ cells. As expected, the association could not be reconstituted if I3/2 immunoprecipitates were prepared from the mock-transfected cells. These data clearly demonstrate that the interaction of p116 and CD45 occurs independent of the isoform status of CD45, a finding consistent with the observation that the 116-kDa protein is detected in CD45 immunoprecipitates prepared from a variety of different cell types expressing different isoforms of CD45. Moreover, the fact that the association can be reconstituted *in vitro* by mixing bead-immobilized CD45 with SAKR/CD45$^-$ cell extracts indicates that p116 is expressed in CD45-negative cells.

Surprisingly, when I3/2 immunoprecipitates from various ψ2 cells were examined prior to the reconstitution of binding assay, a 116-kDa protein was found to be present exclusively in CD45-positive immunoprecipitates (Fig. 4-4*B*). It therefore appears that p116 is ubiquitously expressed, even in cells that do not normally produce CD45. Since CD45 and p116 are constitutively associated in the CD45-expressing ψ2 cells, the ability of CD45 from these cells to bind radiolabeled p116 from SAKR/CD45$^-$ cell lysates during the previous reconstitution assay raises the possibility that not all available CD45 sites are occupied by p116 in the ψ2 cells. Alternatively, it is possible that a certain amount of competition and exchange occur *in vitro*. Since the immunoprecipitates in Fig. 4-4*B* were washed five times in high salt lysis buffer containing 0.5 M NaCl, it would appear that the interaction between CD45 and p116 is refractory to dissociation.

Characterization of p116. Because the association between SAKR-derived CD45 and p116 from SAKR/CD45$^-$ cells could be reconstituted *in vitro*, it was possible address whether p116 is a membrane-bound or cytosolic protein. Membrane and cytosolic fractions were prepared from SAKR/CD45$^-$ cells in which p116 is expressed in the absence of CD45 and is therefore not susceptible to having its subcellular localization influenced by this interaction. CD45 immunoaffinity purified from SAKR cells was mixed with I3/2 beads along with the NP-40-solubilized membranes that either contained (fraction 1) or lacked (fraction 2) detergent-insoluble proteins. Alternatively, the mixing experiment was performed using the cytosolic fraction isolated from these cells. The beads were then washed and subjected to SDS-PAGE. The results of a representative experiment are presented in Fig. 4-5. It can be seen that a 116-kDa protein becomes associated with CD45 after mixing with either of the NP-40-solubized membrane fractions but not the cytosolic fraction from CD45-negative cells. Moreover, depletion of detergent-insoluble material from the membrane fraction does not appear to deplete the 116-kDa protein, suggesting that p116 is a membrane-bound protein and not a component of the cellular cytoskeleton.

To determine whether p116 contains an extracellular domain, healthy SAKR cells were subjected to cell-surface biotinylation or cell-surface radio-iodination. Immunoprecipitated CD45 was strongly labeled under both conditions, while a coimmunoprecipitated 116-kDa protein also exhibited labeling (Fig. 4-6*A* & *B*). Control immunoprecipitates were performed on intracellular proteins, for example p56^{lck}, to ensure that cell membranes did not permit passage of the labeling agents (Fig. 4-6*B* and data not shown). Protein staining of parallel immunoprecipitates run on the same gels demonstrated that the labeled 116-kDa protein comigrates with p116 (Fig. 4-6*A* and data

not shown). For an unknown reason, labeling of the 116-kDa protein by ^{125}I was reproducibly found to be relatively poor in comparison to a higher molecular weight protein that may represent a proteolytic breakdown product of CD45. Collectively, these data suggest that p116 possesses an extracellular domain.

Since CD45 is a PTP, the determination of the phosphorylation status of p116 is significant. By anti-phosphotyrosine immunoblotting, it was found that a CD45-associated protein that comigrates with p116 is tyrosine phosphorylated (Fig. 4-6*A*). It would therefore appear that p116 is capable of becoming tyrosine phosphorylated in T cells.

To ascertain whether p116 is a glycoprotein, CD45 immunoprecipitates containing p116 were incubated with Endo F, which cleaves high mannose and complex *N*-glycans. As shown in Fig. 4-6*C*, there is a shift in the mobility of CD45 which serves as an internal control and a slight shift in the mobility of p116, suggesting that p116 is a glycoprotein. Interestingly, the association between p116 and CD45 is stable following Endo F digestion in that it is not disrupted by washing in NP-40 lysis buffer, indicating that *N*-glycans may not be required to sustain the interaction between these proteins (Fig. 4-7*C*). Altogether, the results of these biochemical experiments suggest that p116 is a surface expressed transmembrane glycoprotein.

C. Summary of Results

The purpose of the present study was to further characterize CD45 function by identifying molecule(s) that specifically associate with CD45. A 116-kDa protein (p116) has been detected in immunoprecipitates from CD45-positive cells but not CD45-

negative T lymphoma cells. The 116-kDa protein appears to be ubiquitously expressed and the association between CD45 and p116 occurs in a variety of different hematopoietic cell types. The interaction between p116 and CD45 can be reconstituted by mixing lysates from CD45-negative cells with purified CD45. The 116-kDa protein associates with isotypically invariant epitopes on the external, transmembrane, and/or membrane-proximal portion(s) of CD45. Results from experiments employing a variety of biochemical approaches suggest that p116 is a cell surface tyrosine-phosphorylated transmembrane glycoprotein.

D. Discussion

Unusual aspects of the association between CD45 and p116. Unlike many previous proteins that have been shown to be associated with CD45, the interaction between p116 and CD45 appears to be highly stable. The association is maintained in the absence of chemical cross-linkers in buffer containing NP-40 and 0.5 M NaCl. Moreover, the association is preserved during overnight incubation at 37°C in the presence of Endo F. The association between p116 and CD45 is not restricted to T cells but occurs in a variety of cell types. Intriguingly, p116 is also detected in non-hematopoietic cells, suggesting that it ubiquitously expressed. Because of its broad distribution, it is possible that identification of p116 will provide additional insights into the function of CD45 in lymphoid as well as non-lymphoid hematopoietic cells.

Identity of p116. Two observations have allowed the elimination of the possibility that p116 is a degradation product of CD45. First, antiserum to the

cytoplasmic domain of CD45 fails to react with p116 by Western blotting. Second, CD45-negative cells express p116 as demonstrated by the reconstitution of binding experiments.

Western blotting experiments carried out using Ab reagents to proteins of similar molecular mass to p116 have yielded negative results (data not shown). Therefore, the functions of this widely expressed CD45-associated protein remain a matter for speculation. One possibility is that p116 regulates CD45 enzymatic activity either directly or by promoting or inhibiting substrate interactions. Given that p116 is tyrosine phosphorylated, it could be a direct substrate of CD45 and therefore a downstream mediator of CD45-triggered signaling events. However, no evidence has yet been obtained to indicate that the phosphorylation of p116 changes during T-cell activation. Since CD45 may interact with the external domain of CD45, the intriguing possibility exists that it may function as a specific ligand. Identification and further characterization of p116 are necessary to clarify its relevance to CD45 functions in lymphocytes and other hematopoietic cells.

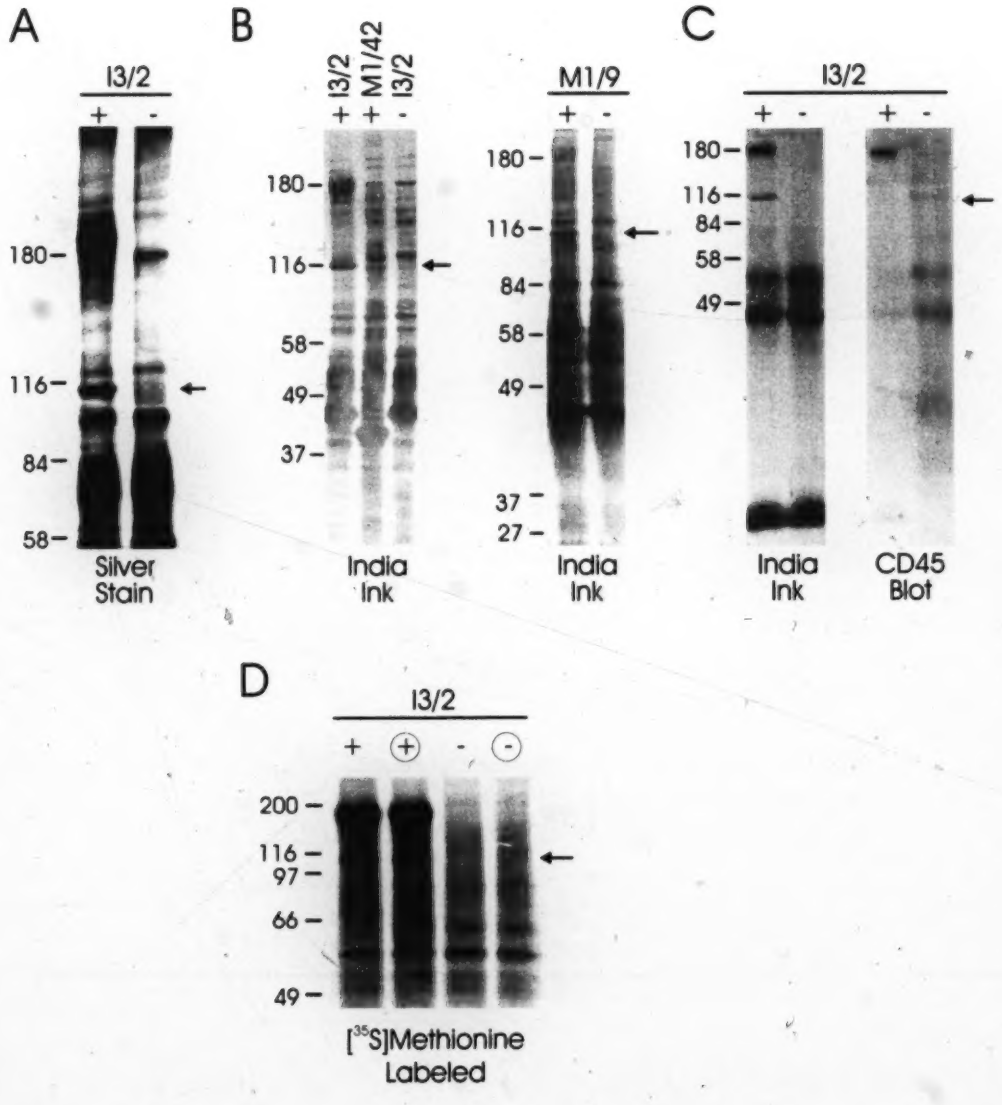

FIGURE 4-1. SDS-PAGE of CD45 immunoprecipitates from CD45$^+$ and CD45$^-$ SAKR cells. I/32 (anti-CD45), M1/9 (anti-CD45), and M1/42 (anti-MHC class I) immunoprecipitates were prepared from NP-40 lysates of SAKR (+) and SAKR/CD45$^-$ (-) cells. *A*, silver-stained gel of immunoprecipitates from 4 x 10^7 cells. *B*, proteins were detected by India ink staining of Immobilon-P transfers and *C*, by Western blotting with anti-95K for detection of CD45 by colorometric assay. *D*, immunoprecipitates from cells metabolically labeled with [^{35}S]Met and pretreated for 10 min with the calcium ionophore ionomycin (*circled*) or with vehicle alone (*not circled*) prior to lysis. *Side arrows* identify the 116-kDa CD45-associated protein.

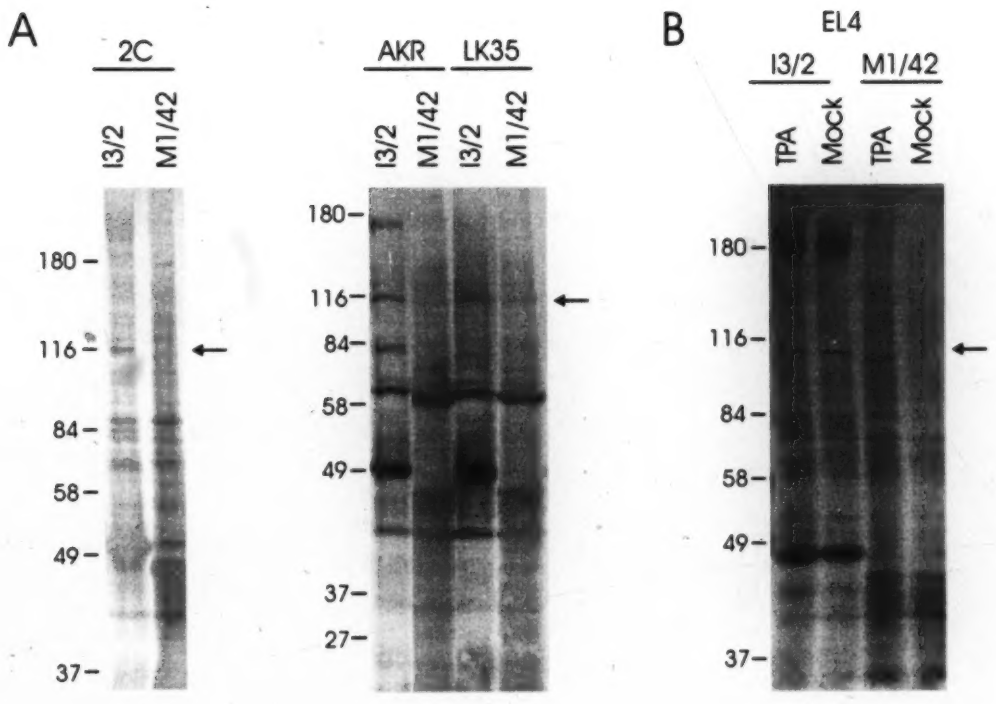

FIGURE 4-2. The association between CD45 and p116 occurs in multiple cell types. *A*, CD45 (*I3/2*) or class I (*M1/42*) immunoprecipitates were prepared from various cell lines, as indicated. Proteins were resolved by SDS-PAGE, transferred to Immobilon-P, and detected by India ink staining. *B*, as in *A* except EL4 cells were lysed following a 10-min pretreatment with TPA or vehicle alone (*mock*). *Side arrows* denote the location of p116.

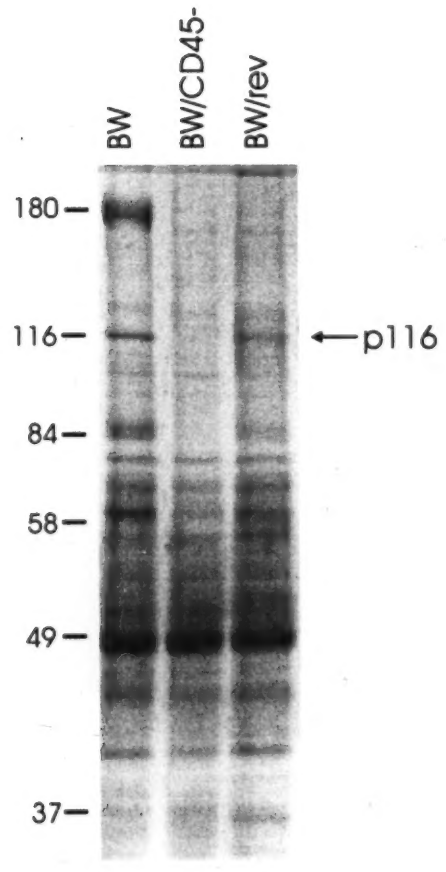

FIGURE 4-3. The 116-kDa protein associates with a mutant form of CD45 lacking most of the cytoplasmic domain. India ink staining of anti-CD45 immunoprecipitates prepared from BW parent cells, a $CD45^-$ variant (*BW/CD45*), and a revertant (*BW/rev*) expressing CD45 with a truncated cytoplasmic domain.

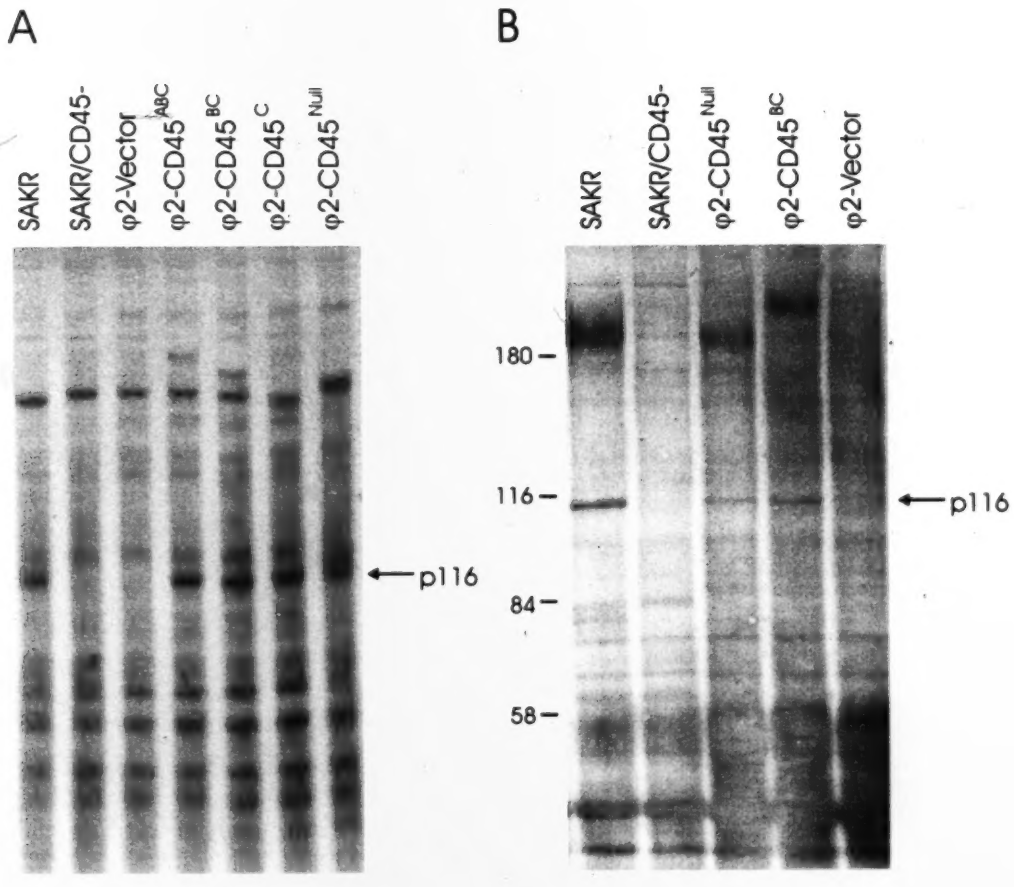

FIGURE 4-4. Association of p116 with individual isoforms of CD45. *A,* I3/2 immunoprecipitates were prepared from φ2 cells expressing $CD45^{ABC}$, $CD45^{BC}$, $CD45^{C}$, $CD45^{Null}$, or containing empty vector. Immunoprecipitates were also prepared from $CD45^{+}$ and $CD45^{-}$ SAKR cells. The immunoprecipitates were washed in lysis buffer and mixed with lysates from SAKR/$CD45^{-}$ cells metabolically labeled with [^{35}S]Met. After a 60-min incubation at 4°C, the immunoprecipitates were washed and subjected to SDS-PAGE followed by autoradiography. *B,* CD45 was immunoprecipitated from a subset of the cells described in *A*. Immunoprecipitates were washed 5 times in high salt wash buffer, and proteins were visualized by India ink staining subsequent to SDS-PAGE.

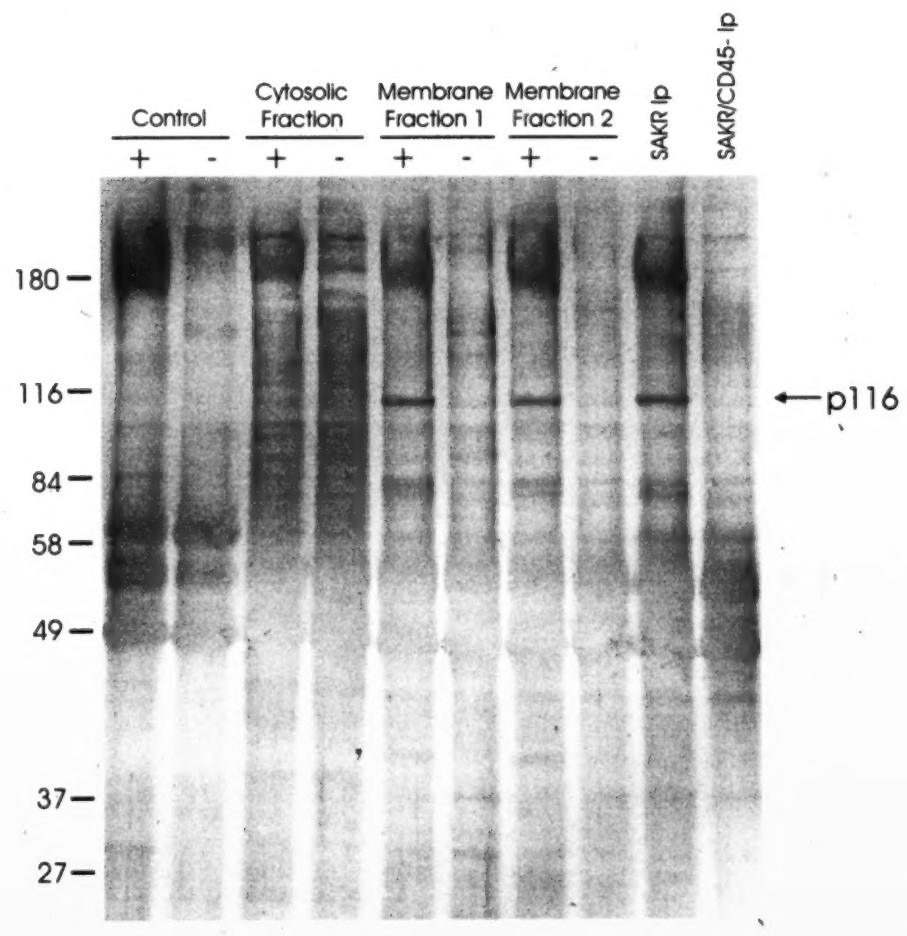

FIGURE 4-5. CD45 associates with a 116-kDa protein found in membranes isolated from CD45-negative cells. The cytosolic fraction, NP-40-solubilized membrane fraction (*membrane fraction 1*), or NP-40-solubilized membrane fraction depleted of detergent-insoluble material (*membrane fraction 2*) prepared from SAKR/CD45$^-$ cells were mixed with I3/2 beads in the presence (+) or absence (-) of purified CD45. As a control, beads and purified CD45 were incubated with lysis buffer rather than cell extracts (*control*). I3/2 immunoprecipitates (*ip*) from 4 x 10^7 CD45$^+$ and CD45$^-$ SAKR cells are also shown. Immunoprecipitated proteins were visualized by India ink staining of proteins transferred to Immobilon-P.

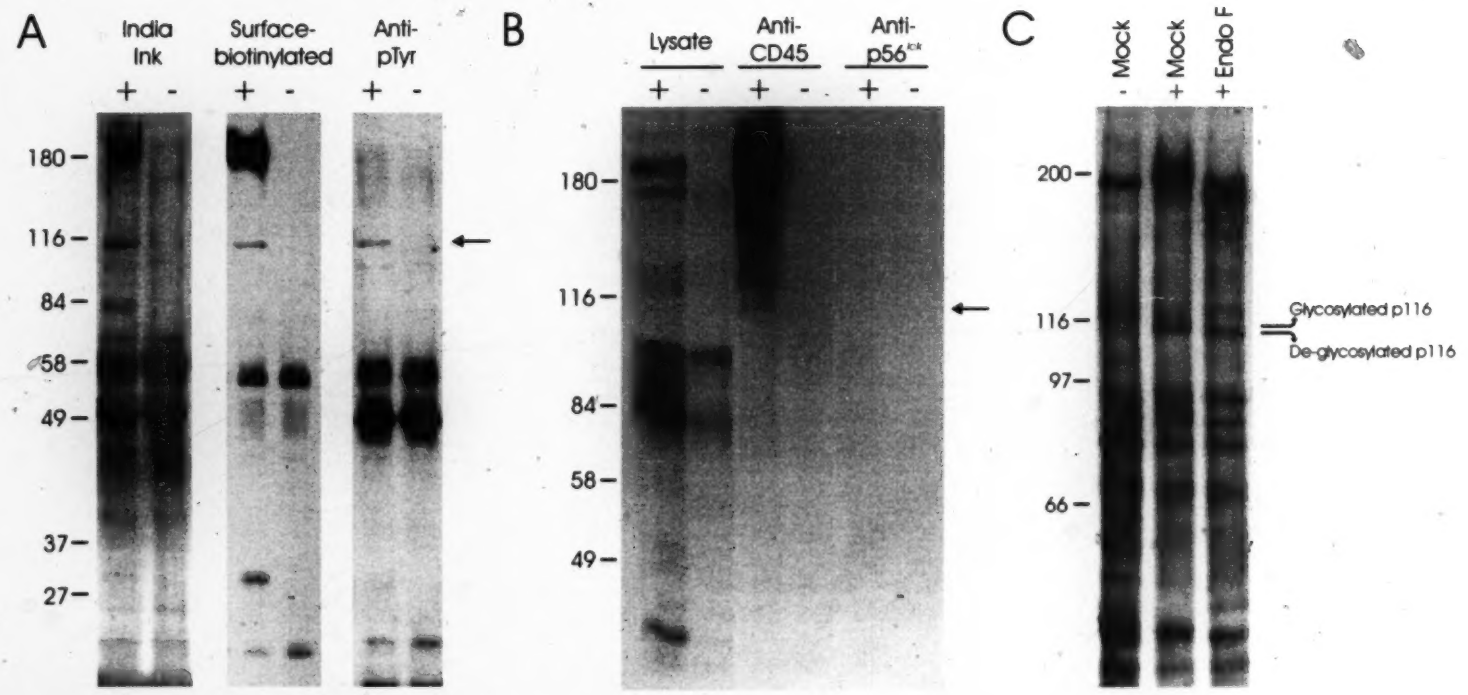

FIGURE 4-6. p116 is a tyrosine-phosphorylated transmembrane glycoprotein. CD45 was immunoprecipitated from $CD45^+$ (+) and $CD45^-$ (-) SAKR cells. *A*, I3/2 immunoprecipitates were prepared in triplicate from surface-biotinylated cells. Samples were loaded onto the same gel for SDS-PAGE and transferred to Immobilon-P for detection by India ink staining or blotting with either streptavidin or anti-phosphotyrosine antiserum. *B*, Cells were subjected to surface iodination prior to immunoprecipitation of CD45 or $p56^{lck}$. Post-nuclear extracts from 10^6 labeled cells are also shown. Radiolabeled proteins were detected by autoradiography. *C*, I3/2 immunoprecipitates prepared from [^{35}S]Met-labeled cells were treated with Endo F or mock-treated overnight at 37°C. The immunoprecipitates were then washed 3 times with lysis buffer and subjected to SDS-PAGE followed by fluorography. *Side arrows* identify the position of p116.

CHAPTER V

IDENTIFICATION OF THE CD45-ASSOCIATED 116-kDa AND 80-kDa PROTEINS AS THE α- AND β-SUBUNITS OF α-GLUCOSIDASE II

(A version of this chapter has been published. Arendt, C. W. and Ostergaard, H.L. 1997. Journal of Biological Chemistry. 272(20): 13117-13125.)

A. Introduction

One means through which insight can potentially be gained into the regulation and function of CD45 is to identify molecules with which it physically interacts. A 116-kDa glycoprotein of unknown identity has been found to specifically associate with CD45 in a wide variety of hematopoietic cells (Arendt and Ostergaard, 1995). The goal of the present study is to purify and identify this protein.

B. Results

Purification of CD45-associated proteins. Association of the 116-kDa protein (p116) with CD45 (Arendt and Ostergaard, 1995) was found to be stable in 0.5% NP-40 but not in 0.5% DOC (Fig. 5-1). Interestingly, in a number of experiments, a protein with an apparent molecular mass of approximately 80 kDa (p80) was also observed in CD45 immunoprecipitates, though non-specific protein bands frequently obscured it. The

association of p80 with CD45 also appeared to be DOC-sensitive (Fig. 5-1). Since neither p80 nor p116 reacted with Abs to known molecules, an attempt was made to purify these proteins by exploiting the differential detergent stability of their association with CD45. Postnuclear supernatants from more than 10^{10} SAKR mouse T-lymphoma cells were prepared in 0.5% NP-40 lysis buffer and passed through an immunoaffinity column containing the I3/2, the pan-specific anti-CD45 Ab routinely employed in previous immunoprecipitation assays. After extensive washing in high-salt (0.5 M NaCl) buffer, fractions were eluted in 0.5% DOC. SDS-PAGE analysis of these fractions by total protein staining revealed two major components migrating at 116 kDa and 80 kDa and additional minor species (Fig. 5-2*A*). This purification scheme was repeated a total of 24 times with highly reproducible results. To control for non-specific protein associations, lysates from an equivalent number of cells from a CD45-negative variant of SAKR (SAKR/CD45$^-$) were processed in an identical manner. Analysis of column eluates from these experiments revealed only faintly staining minor species, indicating that p80 and p116 are physically complexed with CD45 (Fig. 5-2*B*). As a further control, it was confirmed that p80 and p116 were not retained in an anti-class I MHC molecule (M1/42) immunoaffinity column (Fig. 5-2*C*). Thus, 80- and 116-kDa proteins can be copurified on the basis of their stable and specific interaction with CD45.

Microsequence analysis and cDNA cloning of p116 identifies it as the putative catalytic subunit of glucosidase II. Purified p116 and p80 were next subjected to amino-terminal microsequence analysis to obtain sequence information with which to search the BLAST database (Altschul et al., 1990). Sequences from independently-prepared samples of p116 (Table 5-1, samples 1-2) revealed high homology to an incomplete open

reading frame (ORF), GenBank accession number D42041, that had been randomly isolated from the human myeloid cell line KG1 (Nagase et al., 1995). This ORF predicts a putative signal sequence immediately upstream of the corresponding amino-terminus of p116 but is lacking an ATG start codon. Seeking further evidence that p116 is the mouse homologue of this molecule, internal amino acid sequence information was obtained from peptide fragments generated by CNBr digestion (Fig. 5-3). This internal sequence data closely matched the protein sequence predicted by the human ORF (Table 5-1, samples 3-7) with the exception of one peptide fragment (sample 4) that could only be partially aligned with the human protein. Intriguingly, the D42041 ORF recently has had a function ascribed it as the catalytic α-subunit of glucosidase II (GII_α), an α1,3-glucosyl hydrolase of the endoplasmic reticulum (ER) lumen, based on extensive microsequence analysis of a 110-kDa rat protein with glucosidase activity and deletion of a homologous gene in *Saccharomyces cerevisiae* (Trombetta et al., 1996).

Given that a full-length cDNA for GII_α had not been isolated in mammalian cells, and to confirm the identity of p116 as the mouse homologue of this molecule, the cloning and sequencing of the mouse cDNA were undertaken. Several degenerate oligonucleotide primers were synthesized based on the mouse amino acid sequence data. Two of these amplified a 1.9-kb RT-PCR fragment from SAKR cDNA. Cloning and partial sequencing of this amplicon revealed high homology to D42041. Probing of an oligo(dT)-primed mouse T-cell cDNA library with this PCR product led to the isolation of two clones, each 2.9 kb in size, which appeared, from restriction analysis, to be derived from the same mRNA. One of these was sequenced in entirety and found to encode an ORF highly homologous to human D42021, but lacking over 1 kb of 5' sequence. Two rounds of PCR-RACE led to the isolation of a cDNA fragment that contained 11 bases in

the 5'-untranslated region. The complete nucleotide sequence has been submitted to the GenBank database with accession number U92793.

The predicted mass of the protein encoded by this mouse ORF (109 kDa) and the fact that the amino acid sequence information matches that predicted by the nucleotide sequence at all but a single residue (Table 5-1) strongly argue that the cDNA obtained encodes the 116-kDa protein co-purified with CD45. Shown in Fig. 5-4 in alignment with human ORF D42041 (with which it shares 90% amino acid sequence identity), the product of the cDNA contains a long, hydrophobic leader peptide that is cleaved at Ala-32, based on the p116 amino-terminal sequence data and amino-terminal sequence analysis of the rat homologue (Trombetta et al., 1996). A stretch of 22 amino acids spanning residues 187 to 208 in the mouse protein is absent in the human coding sequence, explaining the partial match obtained with the microsequence data in this region (Table 1-5, sample 4). Consistent with previous biochemical analysis of p116 indicating the presence of limited *N*-linked glycans (Arendt and Ostergaard, 1995), a single *N*-glycosylation consensus sequence is present in the mouse protein. Notable also is a conspicuous 21 amino-acid hydrophobic stretch interrupted by several charged residues that, based on biochemical data from a number of sources, does not function as a transmembrane domain (Michael and Kornfeld, 1980; Reitman et al., 1982; Brada and Dubach, 1984; Strous et al., 1987; Trombetta et al., 1996). The high similarity between the protein specified by the mouse cDNA and a partially sequenced 110-kDa rat protein that evidently possesses glucosidase II catalytic function (Trombetta et al., 1996), suggest that p116 represents the α-subunit of glucosidase II.

Glucosidase II α-subunit possesses regions of homology to other α-glucosidases. Database analysis of mouse and human GII_α protein sequences revealed regions of considerable homology to Family 31 glucosyl hydrolases. Contained within this family are a number of ancestrally related gene products with different substrate specificities, including the intestinal sucrase-isomaltase complex and lysosomal α-glucosidase (LAG) (Henrissat, 1991; Henrissat and Bairoch, 1993). Shown in Fig. 5-4 is a portion of the carboxyl-terminal sequence of human LAG (Hoefsloot et al., 1988) aligned with mouse and human GII_α. A significant degree of homology exists between the aligned regions of these proteins, with 36% sequence identity between human LAG and mouse GII_α. In contrast, the remaining amino- and carboxyl-terminal portions of LAG (amino acids 1-477 and 777-952) cannot be aligned with GII_α sequences. Of particular interest is the conservation of a number of residues in the region surrounding Asp-518 in LAG (Fig. 5-4, residues 514-521). Asp-518 has been identified by mutagenesis as the catalytic nucleophile of LAG, while Trp-516 has also been shown to be essential for catalytic function (Hermans et al., 1991). These residues, together with flanking sequences, define a catalytic consensus motif for Family 31 hydrolases: (G/F)-(L/I/V/M)-W-x-**D**-M-N-E (Henrissat, 1991; Hermans et al., 1991; Hoefsloot et al., 1988; Kinsella et al., 1991; Naim et al., 1991; Quaroni and Semenza, 1976; Sugimoto and Suzuki, 1996). As can been seen from the alignments in Fig. 5-4, human GII_α shares the entire catalytic consensus sequence of LAG, while the mouse sequence differs by a conservative substitution of Tyr for Phe at position 560. Therefore, it is possible that this conserved region, shared among a number of apparently related genes encoding

functionally divergent α-glucosidases, represents the active site of the molecule, while other non-conserved sequences may assist in directing enzyme specificity.

Given that the catalytic consensus motif of Family 31 glucosidases is conserved in mouse and human glucosidase II, the question arises as to whether this sequence is also conserved in forms of glucosidase II expressed by other organisms. A number of DNA sequences have recently been deposited in the GenBank and EMBL databases that encode putative glucosidase II homologues in a variety of eukaryotic organisms, ranging from higher mammals (e.g. pig) to lower eukaryotes (e.g. yeast and slime mold) to plants (potato). As summarized in Table 5-2, a striking degree of homology exists between these proteins within a 21 amino acid stretch that spans the putative catalytic site. Astoundingly, the mouse and potato sequences differ only at a single residue (a conservative substitution) within this region. Comparison of the sequences presented in Table 5-2 allows prediction of a catalytic consensus sequence for the glucosidase II family of homologues: N-L-(F/Y)-(I/V)-W-N-**D**-M-N-E-P-S-v-F-n-G-P-E-v-t-m (where uppercase letters indicate completely conserved residues and lowercase letters indicate residues highly favored). It is possible that the relatively large size of this conserved region may relate to the ability of the catalytic site of glucosidase to recognize, in conjunction with the α1,3-linked glucose (Glc) residues that are actually hydrolyzed, branching mannose structures present on immature oligosaccharides (Grinna and Robbins, 1980). The identification of this consensus sequence now allows the design of rational site-directed mutagenesis strategies aimed at elucidating mechanisms of oligosaccharide recognition and catalysis.

Identification and cDNA cloning of the 80-kDa CD45-associated protein corresponding to the putative β-subunit of glucosidase II. Microsequence information derived from the amino terminus of purified p80, in addition to limited sequence data from an internal CNBr-digested peptide fragment (Table 5-3) revealed a close relationship to a human cDNA that was originally cloned as a potential PKC substrate, termed 80K-H (Sakai et al., 1989). More recently, this protein has been proposed to represent the β-subunit of glucosidase II (GII_β), based on its copurification with the α-subunit from rat microsomes and the complete resistance of this complex to biochemical separation (Trombetta et al., 1996). The high degree of sequence conservation between the amino termini of mouse p80 and human 80K-H (Table 5-3) argued in favor of the feasibility of cloning the mouse cDNA by a cross-species hybridization method of library screening. A RT-PCR amplicon corresponding to the entire coding region of human 80K-H was used to screen a mouse T-cell cDNA library. Five independent clones were rescued and their inserts were completely or partially sequenced. All appeared to encode an identical protein exhibiting high homology to 80K-H, though all were missing varying amounts of 5' sequence. PCR-RACE was employed to isolate the 5' end of the cDNA, and PCR cloning of the entire ORF was carried out in SAKR cells. The nucleotide sequence obtained has been submitted to the GenBank database with accession number U92794.

The protein sequence deduced from these cloning experiments, presented in Fig. 5-5, possesses sequences identical to those obtained from microsequence analysis of p80 (Table 5-3). Database analysis of the complete coding region revealed high homology to human 80K-H and an unpublished bovine sequence (GenBank accession number U49178), also shown in Fig. 5-5, but no additional significant primary sequence

homologies to other known proteins. The mouse cDNA encodes a highly acidic 59-kDa protein that lacks a transmembrane domain but possesses a hydrophobic signal sequence that is cleaved at position 14 in mouse (Table 5-3), and at the corresponding site in other species (Sakai et al., 1989; Yang et al., 1991; Goh et al., 1996; Trombetta et al., 1996). The mouse protein shares 86% amino acid identity with its human homologue and 82% identity with the bovine product, with conservation of a number of motifs of potential functional significance. Shared by all species is a carboxyl-terminal His-Asp-Glu-Leu (HDEL) sequence which may serve as an ER retrieval signal by virtue of its ability to interact with a specialized membrane receptor (Ozawa and Muramatsu, 1993; Weis et al., 1994; Wilson et al., 1993). A region containing Glu repeats is also present in all species; similar negatively charged domains are common among ER proteins and may function in ER retention by coordinating low affinity interactions with the Ca^{2+} matrix (Fliegel et al., 1987; Smith and Koch, 1989; Sonnichsen et al., 1994). The unusual charge properties of this region may contribute to the anomalously slow migration of this molecule by SDS-PAGE, as has been observed for similar proteins (Ahluwalia et al., 1992; Sakai et al., 1992; Michalak et al., 1992). In addition to the possible Ca^{2+}-binding activity of the acidic stretch, two EF-hand motifs are present that conform well to known consensus sequences (Marsden et al., 1990) and may confer high affinity Ca^{2+}-binding capacity (Kretsinger, 1980). Interestingly, the more amino-terminal EF hand appears to belong to the troponin family of EF hands, while the second motif can be grouped into the calmodulin family, based on the identity of the third amino acid residues (Marsden et al., 1990). Finally, it is noteworthy that the distribution of Cys residues, clustered near the amino and carboxyl termini of the molecule, is entirely conserved between species, in agreement with evidence for the presence of intrachain disulfide linkages in the rat

homologue (Trombetta et al., 1996). In summary, the second mouse cDNA clone isolated in this study is highly conserved on an evolutionary basis and possesses several hallmarks of an ER protein. The fact that this protein was copurified with the putative catalytic subunit of glucosidase II and the major glycoprotein CD45 is in agreement with a report that, in rat, this 90-kDa protein represents the strongly-associated β-subunit of mouse glucosidase II (Trombetta et al., 1996), a protein of uncertain function.

CD45 immunoprecipitates contain neutral α-glucosidase activity. Given that the CD45-associated proteins encode a carbohydrate-processing enzyme, it was of interest to evaluate the enzymatic activity of the purified complex. Unfortunately, this experiment could not be performed on glucosidase II eluted from the CD45 immunoaffinity column, since it is irreversibly inactivated by DOC (Grinna and Robbins, 1979; Burns and Touster, 1982). Instead, immunoprecipitates prepared from SAKR and SAKR/CD45$^-$ cells were incubated with the chromogenic substrate p-nitrophenyl α-D-glucopyranoside, which is hydrolyzed specifically by glucosidase II at neutral pH (Michael and Kornfeld, 1980). Hydrolysis of the substrate was measured by determining the absorbance at 405 nm. The results of this analysis, presented in Fig. 5-6, reveal that glucosidase II activity is present in I3/2 immunoprecipitates from SAKR, but not SAKR/CD45$^-$ cells. This activity was not detected in anti-class I immunoprecipitates from the same cells. In addition, the activity was abrogated if the assay was performed in an acidic buffer optimized for the evolutionarily related LAG (Jeffrey et al., 1970; Michael and Kornfeld, 1980). It is not clear why active glucosidase II, if functioning

merely in the capacity of a processing enzyme, should remain stably associated with CD45.

Development of antibody reagents specific for glucosidase II. Five unsuccessful attempts were made to generate rabbit antiserum to GII_α by a variety of approaches, including the injection of polyacrylamide gel fragments containing purified and concentrated GII_α. However, two rabbit antisera (termed anti-80.1 and anti-80.2) were successfully raised to distinct regions of GII_β expressed as GST-fusion proteins. These reagents were found to recognize, by Western blot, an 80-kDa protein in both I3/2 immunoprecipitates and p80-positive I3/2 column fractions (Fig. 5-7*A*). Furthermore, both antisera, but not preimmune sera from the corresponding rabbits, were capable of immunoprecipitating an 80-kDa protein from either $CD45^+$ or $CD45^-$ SAKR cells (Fig. 5-7*B*). It was verified that GII_β immunoprecipitated by each Ab reacted with the other Ab by blotting. As an additional control, it was confirmed that immunoreactive protein was absent in M1/42 immunoprecipitates, but present in CD45 immunoprecipitates prepared with the M1/9 mAb (Fig. 5-7*C*), a reagent known also to coprecipitate p116 (GII_α) (Arendt and Ostergaard, 1995). These results convincingly argue that the GII_β cDNA clone encodes a protein identical to that purified by virtue of its ability to form a stable complex with GII_α and CD45.

The association between CD45 and glucosidase II can be reconstituted in vitro. As previously reported, the interaction between CD45 and p116 (GII_α) can be reconstituted *in vitro* by mixing affinity purified CD45 stripped of associated proteins

with lysates from CD45-negative cells (Arendt and Ostergaard, 1995). Since non-specific bands often obscured the 80-kDa region of these protein-stained gels, the reconstitution of binding assay was repeated, making use of the anti-GII_β reagents. As anticipated, it was found that washing CD45 immunoprecipitates with 0.5% DOC led to quantitative release of GII_β that could be sequentially immunoprecipitated with anti-80.2 serum (Fig. 5-8). By incubating CD45-positive immune complexes that had been washed with DOC with lysates from SAKR/CD45$^-$ cells, it was possible to reconstitute the association between CD45 and GII_β. This association could not be reconstituted if CD45-negative beads were incubated with the cell lysates. In conjunction with the previous analysis of p116 (GII_α), these results indicate that the interaction between CD45 and glucosidase II is stable and of high affinity. Moreover, these data exclude the possibility that this association is detected purely as a result of trapping transiently interacting proteins in common detergent micelles upon lysis.

Role of CD45 carbohydrates in mediating the association with glucosidase II. The apparent strength and high stoichiometry of the association between glucosidase II and CD45, and the resistance of this association to disruption in 0.5 M NaCl, were surprising. Although CD45 is an abundant and highly glycosylated protein in T cells, it does not appear to form stable associations were other carbohydrate-processing enzymes or ER folding chaperones under the same conditions that permit copurification of glucosidase II (Fig. 5-2*A*). Furthermore, glucosidase II cannot be detected in stable association with another abundant cell-surface glycoprotein, the class I MHC molecule (Figs. 5-2*C* and 5-7*C*). It was thus of considerable interest to investigate the structural

requirements mediating the association of glucosidase II with CD45. To first eliminate the possibility that the CD45 cytoplasmic domain, which is not exposed to the ER lumen, is required for these proteins to interact, it was confirmed that the association between GII_β and CD45 can occur in the BW/rev cell (Fig. 5-9*A*), which expresses reduced levels of a truncated form of CD45 lacking most of the cytoplasmic domain (Hyman et al., 1982). This is in accordance with the previous observation that the 116-kDa α-subunit also associates with this mutant form of CD45 (Arendt and Ostergaard, 1995).

Next, the reconstitution of binding assay was exploited to address whether CD45 carbohydrate structures are necessary for the association with glucosidase II. CD45 immunoprecipitates that had been washed with DOC to induce dissociation of glucosidase II were incubated with either Endo H (which releases high-mannose type linkages), Endo F (which releases all *N*-linked glycans), or digestion buffer alone. The reconstitution of binding assay was then conducted to assess the ability of GII_β from CD45-negative lysates to become associated with deglycosylated CD45. As is clear from the data presented in Fig. 5-9*B*, the ability to reconstitute the association between GII_β and CD45 was dependent upon the presence of *N*-linked carbohydrates, specifically those of the high-mannose, Endo H-sensitive type. This result indicates that immature oligosaccharide structures present on CD45 are necessary for the association with glucosidase II. Based on the known specificity of glucosidase II, it can be speculated that terminal α1,3-Glc linkages on immature CD45 oligosaccharides are required for binding. In this context, it is intriguing that GII_β does not stably interact with the T-cell protein CD44 (Fig. 5-9*C*), which possesses >30 kDa of *N*-linked glycans (Lesley et al., 1995). It is therefore concluded that *N*-linked glycans, while necessary for the association of

glucosidase II with CD45, are not sufficient to support its stable association with other transmembrane glycoproteins.

Previously, it was reported that the association of CD45 with p116 (GII_α) is stable following Endo F treatment of the intact complex (Arendt and Ostergaard, 1995). In contrast, in the present study, reconstitution of the association of CD45 with GII_β did not occur if Endo F-sensitive glycans were first liberated from CD45. This apparent discrepancy might reflect differences in assay conditions that prevented complete digestion by Endo F in the earlier experiments (e.g. the fact that CD45 was digested in the presence of bound glucosidase II) or differences in the binding requirements of GII_α versus GII_β. Alternatively, it is possible that, similar to the interaction between MHC class I molecules and calnexin (Margolese et al., 1993; Ware et al., 1995), the initiation of glucosidase II binding to CD45 is oligosaccharide-dependent, whereupon highly stable protein-protein interactions are formed that are oligosaccharide-independent.

To address these important issues, Endo F and Endo H digestions were carried out on CD45 in the presence or absence of glucosidase II. Clearly, CD45 is highly Endo F- and H-sensitive regardless of whether glucosidase II is associated (Fig. 5-10*A, compare lanes 5-6 with 8-9*). Intriguingly, though, the presence of glucosidase II was found to confer partial Endo H-resistance to CD45, as revealed by the reconstitution of binding assay (Fig 5-10*B, lane 15*). In this somewhat complex experiment, CD45 digested with Endo H in the presence of glucosidase II, and then released from glucosidase II by DOC wash, was found to be capable of associating with GII_β from CD45-negative lysates. Since the amount of GII_β recruited to bind CD45 under these conditions was substantially less than the level initially bound to CD45 (*lane 9*), it would appear that glucosidase II

provides only limited resistance to Endo H. In contrast, CD45 treated with Endo H in the absence of glucosidase II was completely depleted of immature oligosaccharide structures necessary to allow it to associate with GII_β from CD45-negative lysates (*lane 6*), consistent with the result in Fig. 5-9*B*. The ability of associated glucosidase II to confer partial Endo H resistance to CD45 suggests that its interaction with CD45 occurs with sufficient stability to completely protect a subset of CD45 oligosaccharides from Endo H attack throughout a 16-h digestion at 37°C. This is further confirmed by the finding that, after the Endo H digestion, a portion of GII_β remains stably attached to CD45 as defined by resistance to dissociation following 3 washes with lysis buffer (*lane 12*). Taken together, these data suggest a mode of binding whereby glucosidase II is brought into very close apposition to CD45 oligosaccharide structures.

Interestingly, the association of glucosidase II with CD45 did not impair digestion by Endo F over the course of 16-h reaction period (Fig. 5-10*B, lane 14*). Given that digestion with Endo F proceeded to completion in the presence of glucosidase II, it was possible to more definitively address the question of whether glucosidase II that associates in a carbohydrate-dependent manner with CD45 can remain associated with CD45 following complete removal of *N*-linked glycans. To this end, CD45 was treated with Endo F in the presence of glucosidase II under the same conditions that abrogate the ability of CD45 to form new associations with GII_β in the reconstitution of binding assay. At the end of the digestion reaction, the I3/2 bead fraction was washed 3 times in lysis buffer. As can be seen in Fig. 5-10*B* (*lane 11*), this led to complete loss of GII_β from the bead fraction. Thus, under these *in vitro* conditions, *N*-linked glycans are necessary to preserve the association of CD45 with glucosidase II.

Association of GII_β with multiple CD45 isoforms. The previous data indicate a critical role for Endo H-sensitive glycans on SAKR-derived CD45 in both permitting and preserving the association with GII_β, but do not rule out the possibility that protein-protein interactions might also contribute to the stability of the interaction. It is thus possible that distinct isoforms of CD45, which differ in both protein structure and oligosaccharide content, might differ in their abilities to interact with the β-subunit of glucosidase II, even though previous work indicates that different isoforms of CD45 interact equally well with the α-subunit (Arendt and Ostergaard, 1995). To clarify this issue, YAC-1 cells, which express higher molecular-weight CD45 isoforms than SAKR cells, were examined for the association with GII_β. As shown in Fig. 5-11, GII_β was found to associate with YAC-1-derived CD45. To extend this analysis, I3/2 immunoprecipitates were also prepared from ψ2 cells engineered by Johnson et al. (1989b) to express individual CD45 isoforms. Again, the association between CD45 and GII_β was found to be intact. It is not clear whether this interaction is mediated entirely by the ability of GII_α to recognize specific *N*-linked glycans on CD45, which are located primarily outside of the variable amino-terminal region, or whether GII_β also associates with CD45.

GII_α is required for the interaction of GII_β with CD45. Since GII_β has thus far been found to be associated with CD45 under all conditions that favor the association of GII_α with CD45, it is unclear whether the two proteins are capable of independently associating with CD45. Given that the α- and β-subunits of glucosidase II appear to form a highly stable heterodimer (Trombetta et al., 1996), it would seem most likely that

heterodimeric GII_α/GII_β together bind CD45. However, a better understanding of the means by which glucosidase II stably interacts with CD45 could be achieved if the binding capabilities of each of the two subunits could be dissected. Since purified GII_α and GII_β remain stably associated even the presence of DOC (Trombetta et al., 1996), the question arises as to how to obtain preparations of each subunit in isolation of the other. Fortunately, a mutant cell line was available that allowed this binding issue to be partially addressed. The $PHA^R2.7$ thymic lymphoma is a lectin-resistant variant of BW that is deficient in glucosidase II activity (Reitman et al., 1982). Consistent with the notion that this cell displays abnormal expression of the catalytic subunit of glucosidase II, it was found that only very low levels of reverse-transcribed GII_α transcripts could be amplified from $PHA^R2.7$ by PCR (Fig. 5-12*A*). In contrast, wild-type levels of the full-length GII_β ORF could be amplified from $PHA^R2.7$ cDNA by PCR (Fig. 5-12*A*), and this protein could be directly immunoprecipitated from $PHA^R2.7$ cell lysates (Fig. 5-12*B*), indicating that it is stably expressed. Significantly, while CD45 and GII_β were associated in BW cells, this was not the case in $PHA^R2.7$ (Fig. 5-12*B*). To exclude the possibility that $PHA^R2.7$-dervied CD45 possesses defects that prevent the association with glucosidase II, CD45 immunoprecipitated from the mutant cell was tested in the reconstitution of binding assay. As can be seen from the result in Fig. 5-12*C*, $PHA^R2.7$-derived CD45 is fully capable of associating with GII_β from CD45-negative, glucosidase II-positive cells. This result is not surprising, since the glucosidase II-deficient phenotype of $PHA^R2.7$ should result in the accumulation of high Glc, immature-type oligosaccharide structures on CD45 that are likely to mediate the association with wild-type glucosidase II. Finally, the possibility that $PHA^R2.7$ cells possess a dominant inhibitor to the association between

CD45 and glucosidase II was addressed by performing a lysate mixing experiment. CD45 was immunoprecipitated from a combined lysate consisting of a 1:1 mixture of SAKR and $PHA^R2.7$ cell extracts (Fig 5-12*D*). The amount of GII_β found to coprecipitate with CD45 under this condition was similar to that retrieved from a SAKR cell lysate derived from the same total number of cells, implying that the defect in $PHA^R2.7$ does restrict the ability of SAKR-derived glucosidase II to associate with CD45. Altogether, the above data reveal an essential role for GII_α in facilitating stable binding of GII_β to CD45.

Cell type-specific variability of the association between CD45 and GII$_\beta$. The previous data support a model whereby GII_α and GII_β are capable of stably associating with any CD45 isoform, providing it possesses the appropriate immature *N*-glycan structures necessary to initiate binding. A prediction stemming from this model is that it should be possible to detect GII_β and CD45 in association in all cells expressing this PTP. To test this hypothesis, CD45 was immunoprecipitated from multiple cell types and the association with GII_β was assayed by Western blot. Surprisingly, the interaction of GII_β and CD45 could not be detected in immunoprecipitates prepared from CTL clone AB.1 (Fig. 5-13*A*). This was not due to a defect in expression of GII_β, since it could be both immunoprecipitated and immunoblotted. Furthermore, GII_β from the AB.1 cell lysate was capable of stably associating with SAKR-derived CD45 in the reconstitution of binding assay (Fig. 5-13*B*). These results raise the intriguing possibility that cell type-specific determinants impinge on the ability of glucosidase II and CD45 to stably interact.

All of the previous experiments have employed transformed cell lines or T-cell clones. To examine the status of the association in *ex vivo* cells, thymocytes and

splenocytes were isolated from a variety of inbred mouse strains. Significantly, both subunits of glucosidase II were found to be present in CD45 immunoprecipitates from mouse thymocytes (Fig. 5-14). However, neither subunit could be detected in CD45 immunoprecipitates prepared from splenocytes from a variety of mouse strains. It is unlikely that this dichotomy is due to differences in glucosidase II expression, since GII_β could be immunoprecipitated from splenocytes with efficiency similar to that observed in thymocytes. Together with the previous data, it would appear that the stable association of glucosidase II with CD45 is impaired in certain mature-type cells.

Endo H sensitivity of CD45 derived from immature versus mature cells. It is possible that cell type-specific variation in the association between glucosidase II and CD45 reflects differences in the glycosylation status of CD45. More specifically, CD45 from cells in which the association does not occur may be deficient in immature-type oligosaccharides that are obligatory for glucosidase II binding. An experiment was carried out to assess the immature carbohydrate content of total cellular pools of CD45 expressed in various cell types. As presented in Fig. 5-15, CD45 immunoprecipitated from the BW or PHA^R2.7 thymic lymphoma cells underwent a significant gel mobility shift when treated with Endo H, similar to that seen in earlier experiments involving SAKR cells (5-10*A*). Interestingly, a mobility shift was also observed when clone AB.1-derived CD45 was treated with Endo H, indicating that CD45 also possesses detectable immature *N*-glycans in this mature-type cell. These results are intriguing in that they emphasize that additional factors beyond carbohydrate content may influence the ability of glucosidase II to stably associate with CD45.

C. Summary of Results

Herein it is demonstrated that two proteins of 116 kDa and 80 kDa copurify with CD45 from mouse T cells. Microsequence analysis of the 116-kDa protein reveals high similarity to an incomplete human ORF that has been suggested to correspond to the catalytic α-subunit of glucosidase II. The nucleotide sequence of the mouse cDNA has been determined, and codes for a product nearly identical to its human homologue that shares an active site consensus sequence with related α-glucosidases. Amino acid sequencing of the 80-kDa protein, followed by molecular cloning, reveals high homology to human and bovine cDNAs postulated to encode the β-subunit of glucosidase II. Antisera developed to the mouse β-subunit have been used to demonstrate that the stable interaction of glucosidase II with CD45 is initiated upon recognition by glucosidase II of Endo H-sensitive oligosaccharide linkages on CD45. Interestingly, stably associated glucosidase II protects a subset of CD45 oligosaccharides from attack by Endo H. GII_β associates with CD45 only in the presence of wild-type GII_α, and the association is not altered by differential expression of CD45 isoforms. It is thus surprising that the association between glucosidase II and CD45 exhibits cell type variability, occurring in a variety of immature-type cells including thymocytes but not in CTL clone AB.1 or splenocytes.

D. Discussion

Functions of glucosidase II. Glucosidase II is a neutral pH exo-glucohydrolase of the ER involved in the processing of *N*-linked triantennary core glycans acquired in a co-translational manner by nascent polypeptide chains (Helenius, 1994). Glucosidase II acts in sequence after glucosidase I has cleaved the terminal, α1,2-linked Glc from protein-conjugated $Glc_3Man_9GlcNAc_2$ to hydrolyze the inner two α1,3-linked Glc units (Fig. 5-16). The activity of glucosidase II in the ER is counteracted by that of UDP-glucose:glycoprotein glucosyltransferase (UGGT), which can add back a single Glc linkage to $Man_9GlcNAc_2$. The opposing activities of these two enzymes has been accounted for in a "quality-control" model of ER-processing, whereby incompletely-folded proteins must present a monoglucose moiety to be recognized by the folding chaperones calnexin and calreticulin. This scheme ascribes a dual function to glucosidase II. By removing the first α1,3-linked Glc, glucosidase II allows nascent polypeptides to interact with calnexin/calreticulin, whereas removal of the innermost Glc is accompanied by dissociation from the chaperone and, ultimately, egress from the ER.

Biochemical and molecular identification of glucosidase II. The design of arduous purification protocols allowed several groups to isolate glucosidase II and characterize its activity (Grinna and Robbins, 1979; Ugalde et al., 1979; Michael and Kornfeld, 1980; Ugalde et al., 1980; Burns and Touster, 1982; Brada and Dubach, 1984; Hino and Rothman, 1985). Because glucosidase II was purified based solely on its enzymatic activity in these procedures, variation in results led to disagreements over its

true biochemical identity. Glucosidase II has been purified as a protein with an estimated mass of 65 kDa (Burns and Touster, 1982), 100 kDa (Brada and Dubach, 1984), and 123 kDa (Hino and Rothman, 1985), both in the presence (Brada and Dubach, 1984; Hino and Rothman, 1985) and apparent absence (Burns and Touster, 1982) of a second protein of approximately 90 kDa. Discrepancies in results were attributed to proteolysis, and the 90-kDa protein was dismissed as a breakdown product. Very recently, Trombetta et al. (1996) succeeded in simplifying the purification of glucosidase II from rat liver microsomes and also obtained two proteins (110 kDa and 90 kDa) that could not be further fractionated by any high-resolution chromatographic method short of denaturation. A variety of techniques were employed to deduce that the proteins exist as a heterodimeric complex, and extensive microsequence information was obtained on both members. The mouse homologues of these two proteins have now been copurified with the transmembrane PTP, CD45, in T cells and their corresponding cDNA sequences have been determined.

Glucosidase II α-subunit. GII_{α} is quite unlike most other processing enzymes of the ER and Golgi in that it is a non-transmembrane protein, and as such is completely stable in the absence of detergent (Trombetta et al., 1996). GII_{α} does contain a hydrophobic stretch of approximately 20 amino acids, but the hydrophobic character of this region is reduced by the presence of two polar (both Ser) and one charged (Asp) amino acid. Although glucosidase II was initially identified in membrane fractions (Grinna and Robbins, 1979; Ugalde et al., 1979; Michael and Kornfeld, 1980; Brada and Dubach, 1984), it could be extracted under conditions that do not remove integral membrane proteins (Michael and Kornfeld, 1980; Brada and Dubach, 1984).

Furthermore, it was found to partition primarily (though not exclusively) to the aqueous phase in Triton X-114, thus confirming its identity as a non-integral protein with so-called "ambivalent" characteristics (Strous et al., 1987). The mechanism by which this soluble protein associates with microsomal membranes is not clear, however the tendency of glucosidase II to be retained in membrane fractions assists in clarifying why p116 (GII_α) was previously characterized as a transmembrane protein (Arendt and Ostergaard, 1995).

Glucosidase II β-subunit. The present study supports the surprising finding by Trombetta et al. (1996) that glucosidase II consists of two protein subunits. One function of GII_β may be to couple GII_α to retention and/or retrieval mechanisms such as the transmembrane KDEL receptor, possibly explaining the membrane-partitioning behavior of the enzyme complex. Although GII_β does not appear to be necessary for GII_α catalytic function (Brada and Dubach, 1984; Hino and Rothman, 1985; Kaushal et al., 1993; Trombetta et al., 1996), it may exert a regulatory effect on the activity of GII_α or its substrate specificity. It not clear whether this protein is capable of performing additional functions independent of GII_α. The observation that GII_β cannot associate with CD45 in the absence of wild-type GII_α is consistent with the possibility that GII_β forms protein-protein contacts with GII_α but not CD45. It also possible that GII_β does interact with CD45 but that this interaction is unstable in the absence of GII_α. If GII_β-free preparations of GII_α can be obtained, it will be important to address the reciprocal issue of whether GII_β stabilizes the association of GII_α with CD45. It should be noted that the results of

the present study do exclude the possibility that the conformation of GII_β is impaired in the absence of GII_α in $PHA^R2.7$ cells, thus altering its binding properties.

Mechanism of glucosidase II binding to CD45. It has been clearly demonstrated that oligosaccharides of the Endo H-sensitive type are necessary for the initial interaction between CD45 and glucosidase II. While failing to confirm the existence of protein-protein interactions between CD45 and glucosidase II, the present experiments do not refute the possibility that such interactions occur. It is entirely possible that the initial oligosaccharide-dependent interaction of glucosidase II with CD45 leads to an oligosaccharide-independent protein-protein interaction. However, if the proteins were to dissociate at any time during the 16-h endoglycosidase reaction, their re-association might be prevented by Endo F-mediated elimination of oligosaccharide structures necessary for initiation of binding. Alternatively, it is possible that the binding interaction between CD45 and glucosidase II involves protein-protein interactions that are only stable in the presence of oligosaccharide structures recognized by glucosidase II. Finally, it should be noted that the present data contrast with previous results indicating that the association between p116 (GII_α) is maintained following Endo F treatment (Arendt and Ostergaard, 1995). Although it is not possible to definitively resolve this discrepancy, the simplest explanation is that the Endo F digestions did not proceed to completion in the earlier experiments due to the slightly different assay conditions employed. A more provocative possibility, that GII_α is capable of stably remaining associated with CD45 in the absence of *N*-glycans but GII_β is not, must also be considered. These issues can be addressed with greater confidence when Ab reagents to GII_α become available.

Significance of the association between CD45 and glucosidase II. An important question arising from this study is why it is possible to copurify glucosidase II with CD45. Glucosidase II recognizes an oligosaccharide structure added to all nascent proteins that undergo *N*-linked glycosylation. Since the interaction between glucosidase II and CD45 is dependent on high-mannose triantennary core oligosaccharide glycans of the Endo H-sensitive type, it seems logical that glucosidase II interacts with newly synthesized CD45 containing terminal α1,3-Glc linkages. The simplest explanation for the observed association, then, is that glucosidase II interacts with CD45 as it does with all newly synthesized glycoproteins, however, because CD45 is a highly expressed, multivalent substrate this transient interaction is easily detected. However, this explanation is not entirely satisfying, since all the data indicate that the association occurring between CD45 and glucosidase II is exceptionally stable. Moreover, even low levels of glucosidase II cannot be detected in association with other abundant glycoproteins such as class I MHC molecules or CD44. If the association with glucosidase II is simply a byproduct of the glycosylation status of CD45, it is surprising that similarly high levels of other processing enzymes such as glucosidase I, or ER chaperones such as calnexin/calreticulin, are not obtained during the purification procedure. It is also puzzling as to why it is that the glucosidase II does not stably interact with CD45 in splenocytes or Ag-dependent, IL-2 dependent CTL clone AB.1 cells. Since the CD45 isoforms expressed by these cells are highly glycosylated, they must therefore, by definition, at some time be acted upon by glucosidase II. That the interaction of glucosidase II with CD45 occurs transiently in some cells and stably in others suggests additional regulatory mechanisms that have yet to be elucidated which

may relate to novel aspects of CD45 biogenesis or function. A hypothetical model to account for these observations is presented in the General Discussion (Chapter VIII).

TABLE 5-1
Amino acid microsequence analysis of the 116-kDa CD45-associated protein

No.[a]	Mouse protein sequence[b]	Human D42041 sequence[c]
1	VDRSNFKTxDESsFxkRQ	VDRSNFKTC**E**ESSFCKRQ (32-49)
2	vDRSNFKT	VDRSNFKT (32-39)
3	vDrsnFktxdeS	VDRSNFKTC**E**ES (32-43)
4	MAFEHQRAPRVPFxDKVVLaLGSV[d]	**LE**FEHQRAPRV**SQ**G**S**K**DPAEGDGA** (175-198)
5	Mgagkpaavvl	**I**GAGKPAAVVL (894-904)
6	Mkddpitlfvalspqgt	MKDDPITLFVALSPQGT (822-838)
7	Mkxdxitlfval	MKDDPITLFVAL (822-833)

[a] Samples 1 and 2 were subjected to amino-terminal sequence analysis. Samples 3 to 7 are peptide fragments generated by CNBr digestion. Peptide 3 corresponds to the amino terminus of the 116-kDa protein.

[b] Sequences are denoted in standard single-letter code. Some sequencing cycles yielded multiple peaks; in these cases the major peak is indicated in lowercase type. An "x" has been placed at positions where amino acid identity could not be determined. The identity of the methionine at the first position of peptides 4 to 7 is inferred.

[c] Regions of the human D42021 ORF (GenBank) homologous to those sequenced in the 116-kDa mouse protein are presented. The location of these residues in the human protein is indicated in *parentheses*. *Boldface type* identifies human sequences that do not match those in the mouse protein.

[d] Residue underlined is the only site that does not match the protein sequence predicted by the cDNA clone of the 116-kDa mouse protein.

TABLE 5-2

The region surrounding the putative catalytic site of glucosidase II is highly conserved between mammals and lower eukaryotes

Organism	Sequence[a]	Residues	Database accession number	Function
Mus musculus	N L Y V W N ↓D M N E P S V F N G P E V T M	558-578	U92793	Not known
Homo sapiens	- - F - - - - - - - - - - - - - - - - - -	535-555	D42041	Not known
Rattus norbegicus	- - - - - - - - - - C -[b]	not known[b]	not cloned[b]	α-Glucosidase
Sus scrofa	- -	536-556	U71273	α-Glucosidase
Caenorhabditis elegans	- - H I - - - - - - - - - - S - - - I - -	510-530	Z70753	Not known
Dictyostelium discoideum	- S -	534-554	U72236	Not known
Saccharomyces cerevisiae	- - F I - - - - - - - - I - D - - - T - A	531-551	Z36098	α-Glucosidase
Solanum tuberosum	- - - I - - - - - - - - - - - - - - - - -	510-530	AJ001374	Not known

[a] Residues identical to the mouse sequence are indicated with *dashes*. An *arrow* identifies the putative catalytic nucleophile.

[b] Based on microsequence analysis of purified glucosidase II (Trombetta et al., 1996).

TABLE 5-3

Amino acid microsequence analysis of the 80-kDa CD45-associated protein

No.[a]	Mouse protein sequence[b]	Human 80K-H sequence[c]
1	VeVKRPrGVsl	VEVKRPRGVSL (15-25)
2	vEvKRP	VEVKRP (15-20)
3	VEVKRPxGVSLSNHHFYEESKPFTcLDG TATIPFD	VEVKRPRGVSL**T**NHHFY**D**ESKPFTCLDG **S**ATIPFD (15-49)
4	MkYEQ	MKYEQ (462-466)

[a] Sample 1 was subjected to amino-terminal sequence analysis. Samples 2 to 4 are CNBr-cleaved peptide fragments, with peptides 2 and 3 representing the amino terminus of the 80-kDa protein.

[b] Sequences are presented as outlined in the footnote to Table 5-1. The identity of the methionine at the first position of peptide 4 is inferred.

[c] Regions of the human 80K-H protein (Sakai et al., 1989) homologous to those sequenced in mouse p80 are presented as described in the footnote to Table 5-1.

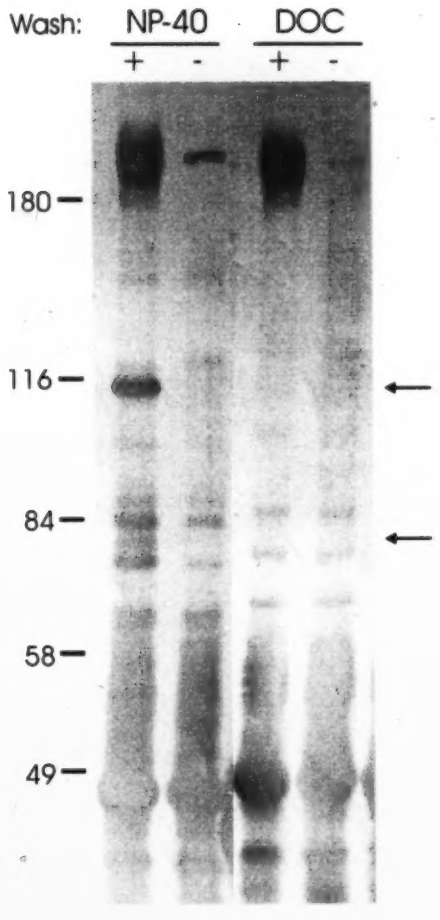

FIGURE 5-1. CD45 association with p116 and p80 is stable in NP-40 but not DOC. I3/2 (anti-CD45) immunoprecipitates prepared from 0.5% NP-40 lysates of CD45-positive (+) and CD45-negative (-) SAKR cells were washed in 0.5% NP-40 or 0.5% DOC. Proteins were separated by SDS-PAGE, transferred to Immobilon-P, and visualized by India ink staining. *Side arrows* indicate the positions of the 116-kDa and 80-kDa CD45-associated proteins.

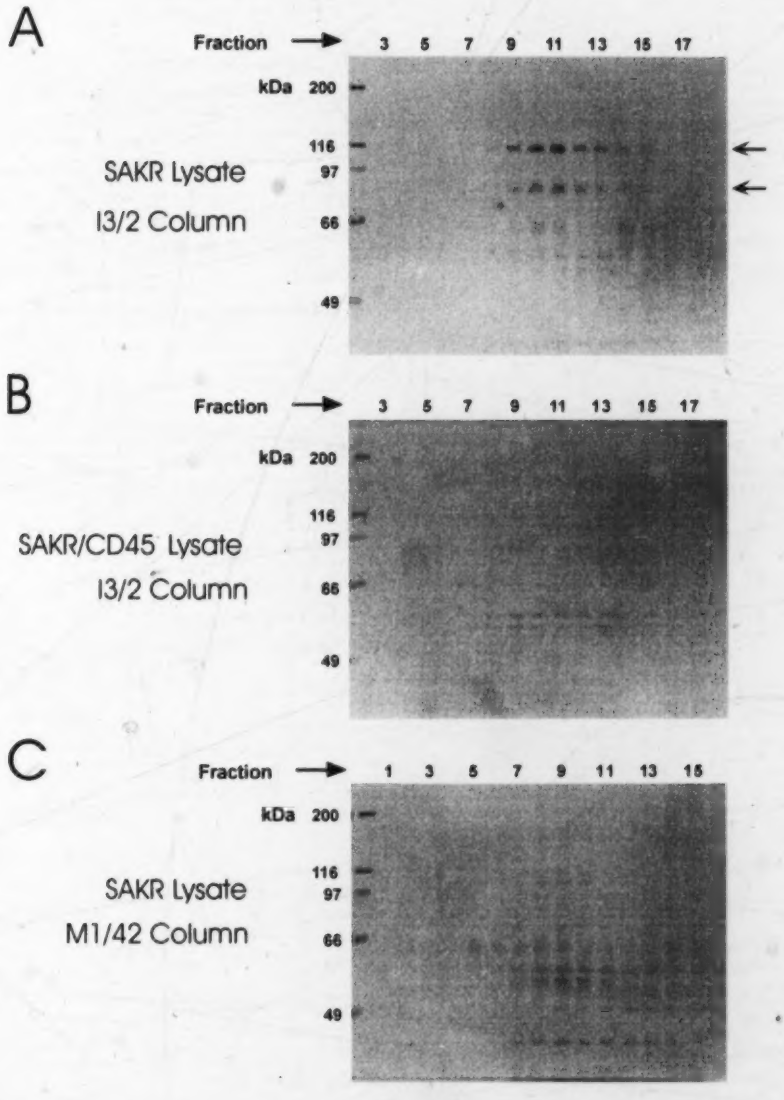

FIGURE 5-2. Immunoaffinity purification of CD45-associated proteins. NP-40 lysates from 1.6 x 10^{10} $CD45^{+}$ (*A* and *C*) or $CD45^{-}$ (*B*) SAKR cells were loaded onto anti-CD45 (*A* and *B*) or anti-MHC class I (*C*) immunoaffinity columns. After washing in buffer containing 0.5 M NaCl, proteins were eluted by addition of buffered 0.5% DOC. Shown are Coomassie Blue-stained gels containing 40 µl from each 1-ml column fraction, compared to 1 µg each of various standard proteins. *Arrows* designate the positions of p116 and p80.

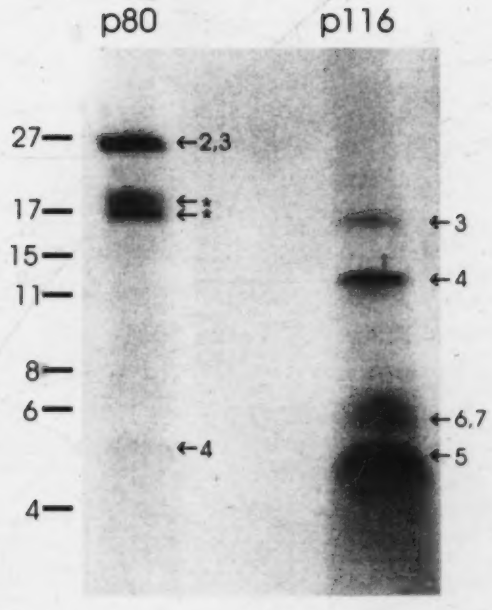

FIGURE 5-3. Electrophoretic analysis of peptide fragments generated from CNBr digestion of p80 and p116. CNBr cleavage products of p80 and p116 were resolved on a 16.5% tris-tricine gel. Proteins were transferred to Immobilon-P^{SQ} for staining with Coomassie Blue dye. Peptides generated in this preliminary experiment as well a larger subsequent experiment were excised for microsequence analysis. The bands are assigned numbers corresponding to the sequence data presented in Tables 5-1 and 5-3. Peptides sequenced in two independent experiments are numbered twice, and peptides for which sequence information could not be obtained are marked with an *asterisk*.

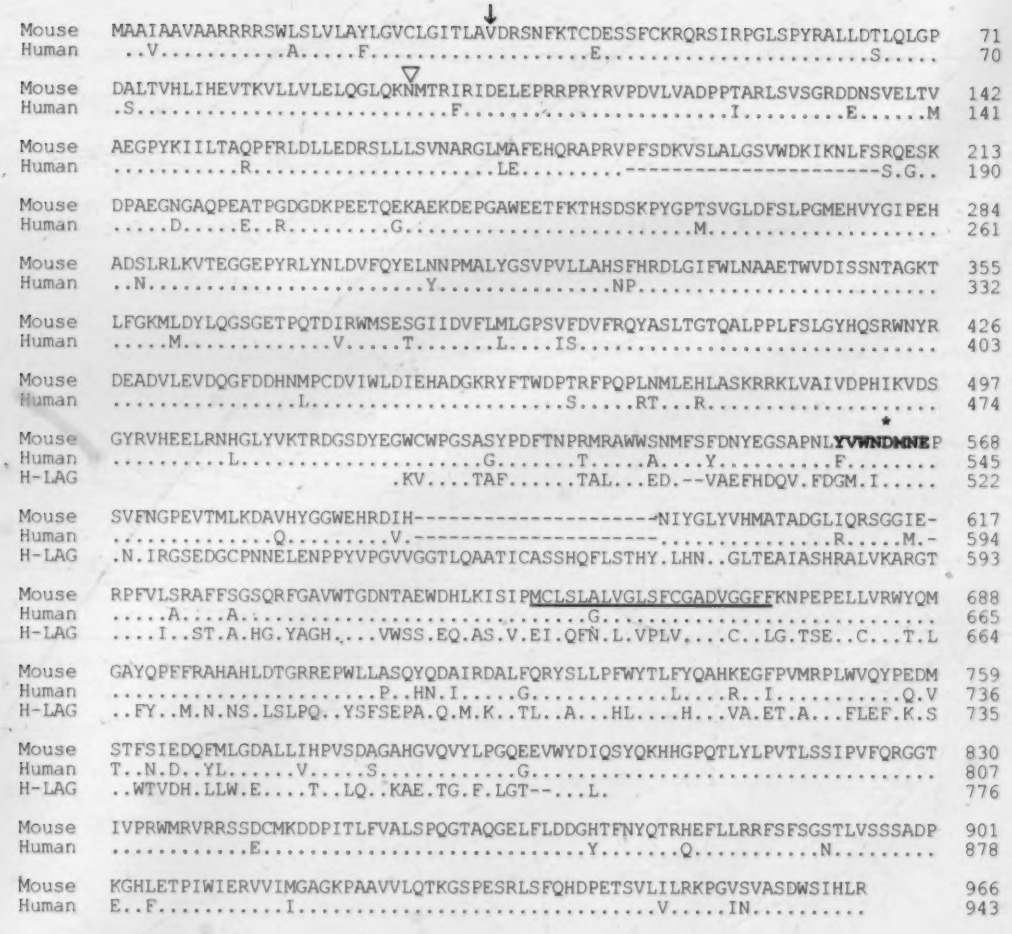

FIGURE 5-4. Amino acid sequence alignment of mouse GII_α with its human homologue and a Family 31 glucosyl hydrolase. The deduced amino acid sequence of mouse GII_α is compared with the predicted sequence of human homologue D42041. Also aligned with these sequences is a portion (amino acids 478 to 776) of human lysosomal α-glucosidase (H-LAG). *Dots* identify residues identical to the mouse sequence; gaps are signified by *hyphens*. An *arrow* marks the amino terminus of the mature protein, and a hydrophobic stretch of amino acids is *underlined*. A *triangular arrowhead* denotes the position of the single Asn-linked glycosylation consensus sequence. The region corresponding to the active site in H-LAG is presented in *boldface type* and an *asterisk* indicates the catalytic nucleophile.

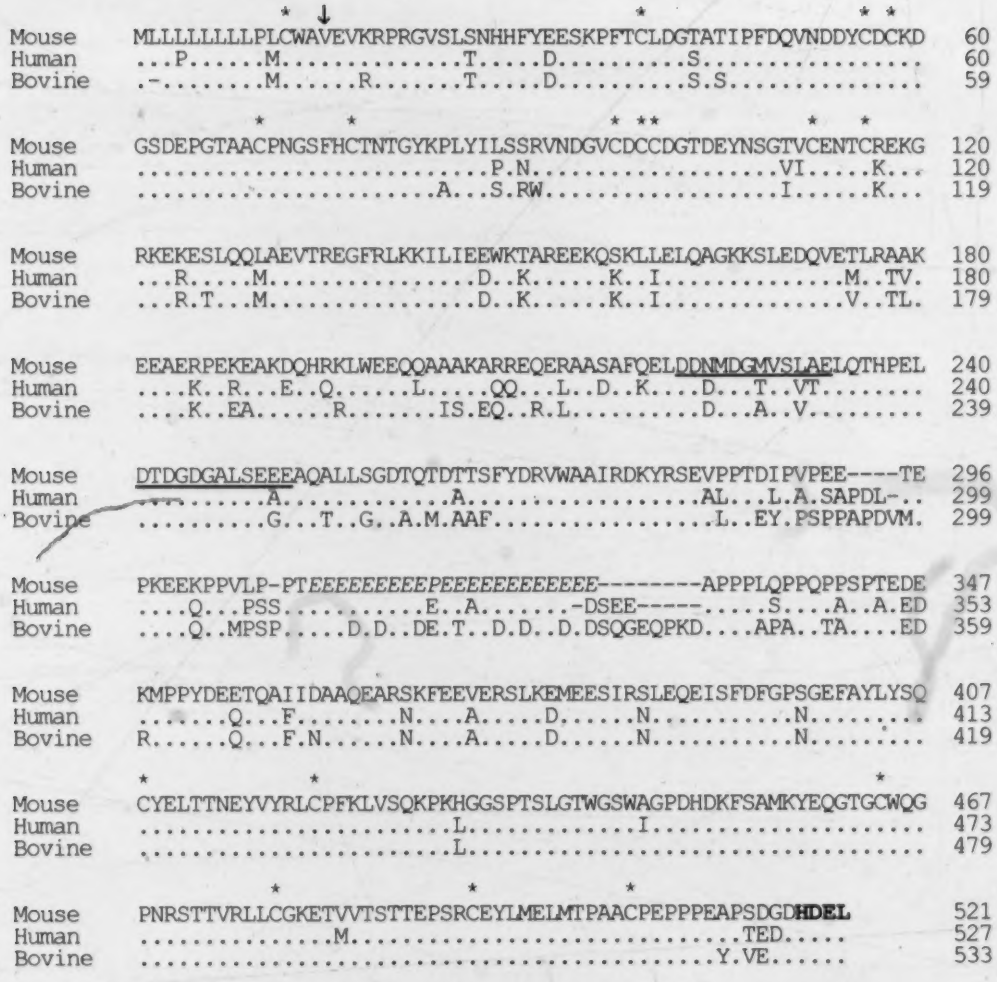

FIGURE 5-5. Comparison of mouse, human, and bovine proteins encoding the putative β-subunit of glucosidase II. Predicted amino acid sequence of mouse p80 is shown together with homologous human and bovine ORFs. Human and bovine sequences matching those in mouse are indicated with *dots*; gaps (–) are marked. An *arrow* identifies the amino terminus of the processed protein. Motifs corresponding to the putative EF-hands are *underlined*, and a highly acidic stretch is presented in *italics*. The carboxyl-terminal HDEL tetrapeptide is in *boldface type*. Cys residues, all of which are conserved among the three species, are demarcated with *asterisks*.

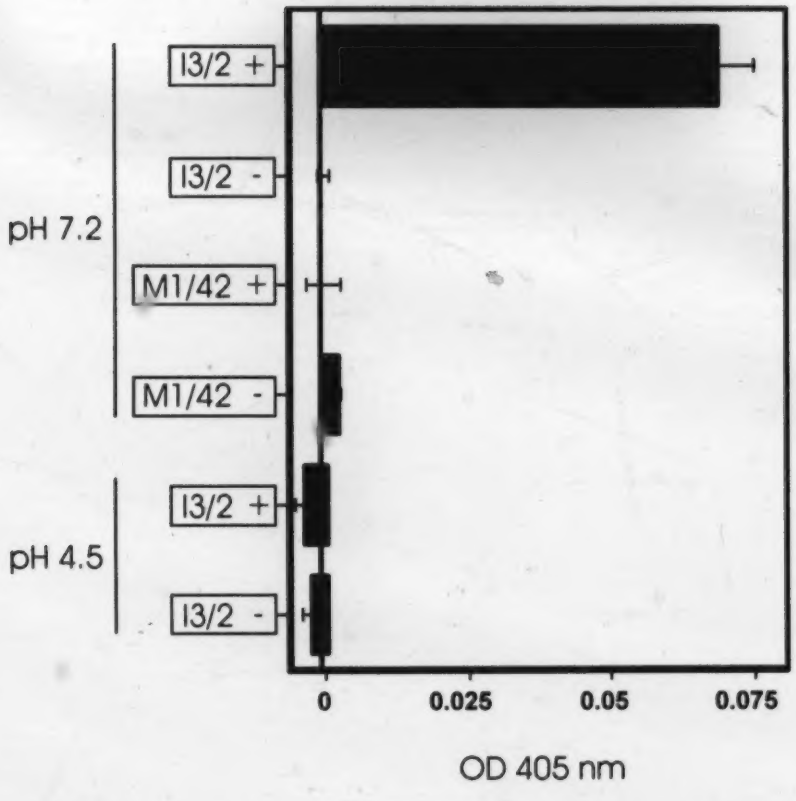

FIGURE 5-6. Neutral α-glucosidase activity is present in CD45 immunoprecipitates. I3/2 and M1/42 immunoprecipitates were prepared from SAKR (+) and SAKR/CD45⁻ (-) cells. After washing the beads in lysis buffer, immune complex-associated α-glucosidase activity was measured by incubating the beads with p-nitrophenyl α-D-glucopyranoside under neutral or acidic pH buffering conditions. The results are expressed as mean OD_{405} values obtained from 3 parallel immunoprecipitates after subtraction of background absorbance.

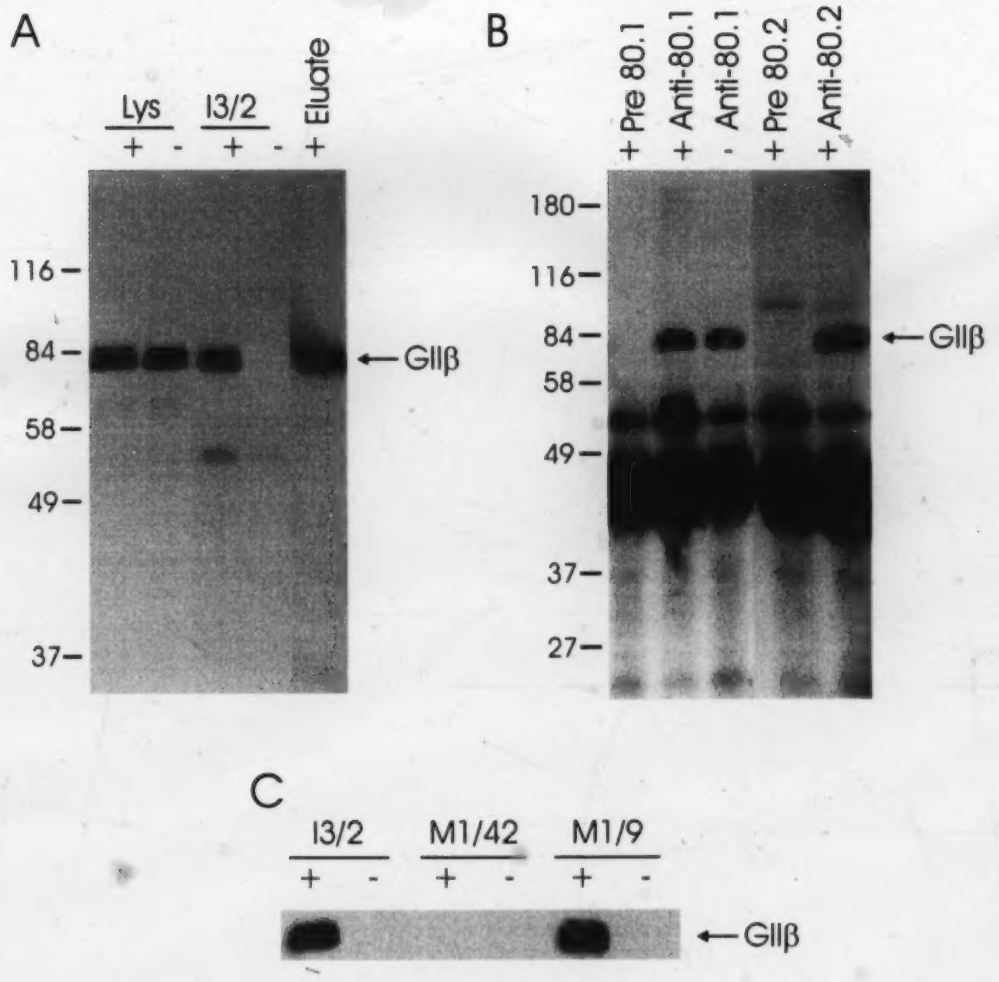

FIGURE 5-7. Reactivity of specific antisera generated against recombinant GII$_\beta$. Rabbit antisera (anti-80.1 and anti-80.2) were generated to two non-overlapping portions of mouse GII$_\beta$ expressed as recombinant GST-fusion proteins. Reactivity of the reagents was tested on CD45$^+$ (+) and CD45$^-$ (-) SAKR cell proteins. *A*, blotting by the anti-80.2 reagent of p80/p116 eluted from the CD45 immunoaffinity column. For comparison, I3/2 immunoprecipitates and 1.25 x 10^6 equivalents of nuclear-free lysates (*Lys*) were loaded onto the same gel. *B*, immunoprecipitates were prepared with the two anti-80 sera or with preimmune sera from the same rabbits. The Western blot was probed with anti-80.1 serum. *C*, immunoprecipitates were prepared using the mAbs indicated. Anti-80.1 serum was employed in Western blot analysis.

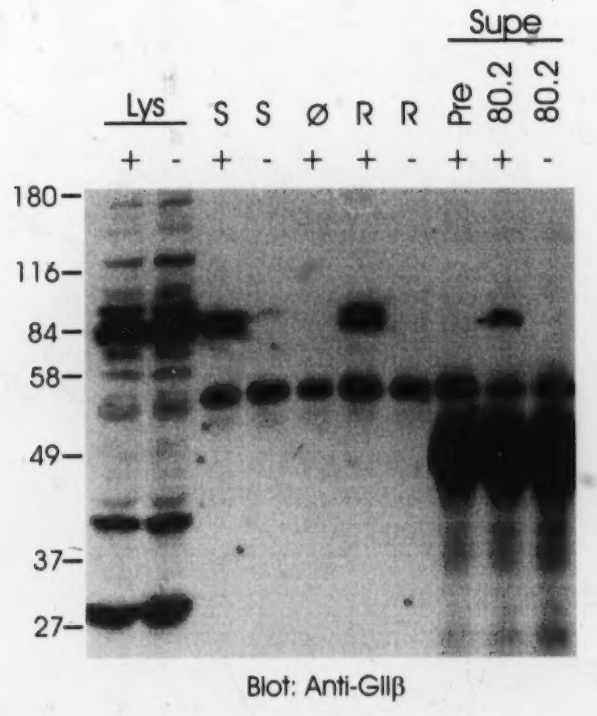

FIGURE 5-8. Binding of GII_β to CD45 can be reconstituted *in vitro*. *A*, CD45 was immunoprecipitated from 5 x 10^7 SAKR (+) or SAKR/CD45$^-$ (-) cells. One sample pair was washed in lysis buffer and boiled immediately in reducing SDS sample buffer (*S*). The experimental group was washed 2 times in dissociation buffer to release GII_α/GII_β from their association with CD45. Immune complexes were then incubated for 2 h with lysates from 5 x 10^7 SAKR/CD45$^-$ cells to test for reconstitution of binding between bead-immobilized CD45 and glucosidase II in the CD45-deficient cell lysate (*R*). To ensure that the washes with dissociation buffer were effective, one sample from the experimental group was boiled immediately in sample buffer following the DOC wash (Ø). As a further control, the supernatant from the first wash with dissociation buffer (*Supe*) was subjected to immunoprecipitation with anti-80.2 serum or preimmune (*Pre*) serum. Finally, lysates (*Lys*) from 10^6 cells were also boiled directly in sample buffer. Following SDS-PAGE, blotting was carried out with anti-80.2 serum.

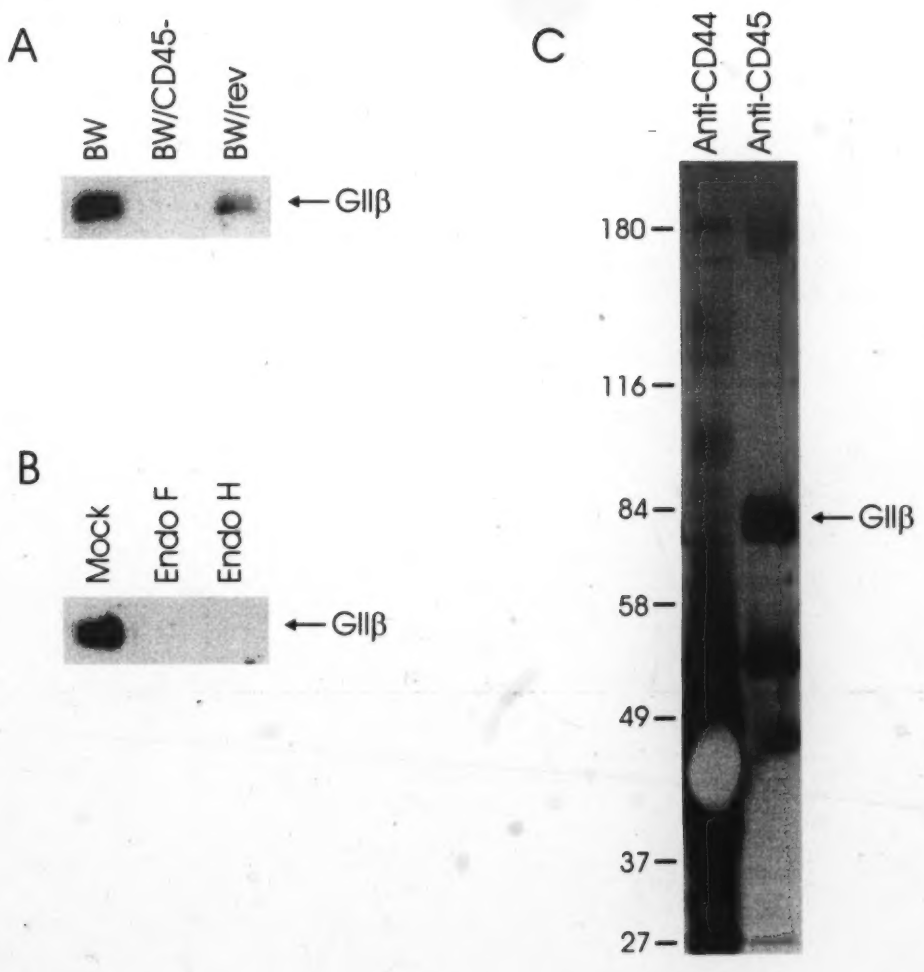

FIGURE 5-9. Structural requirements for GII$_\beta$ binding. *A*, 13/2 immunoprecipitates prepared from BW, BW/CD45$^-$, and BW/rev cell lysates were subjected to immunoblotting with anti-80.2 serum. *B*, CD45 was immunoprecipitated from SAKR lysates and stripped of associated proteins by DOC wash. Immune complexes were then incubated with Endo F, Endo H, or buffer alone (*mock*) for 16 h at 37°C. The ability of deglycosylated CD45 to associate with glucosidase II was assayed by incubating with SAKR/CD45$^-$ cell lysates. Bound GII$_\beta$ was detected by blotting with anti-80.2. *C*, CD44 and CD45 were immunoprecipitated from lysates of the EL4 T-lymphoma. The blot was probed with anti-80.2 serum and intentionally overexposed to show the absence of GII$_\beta$ in the anti-CD44 immunoprecipitate.

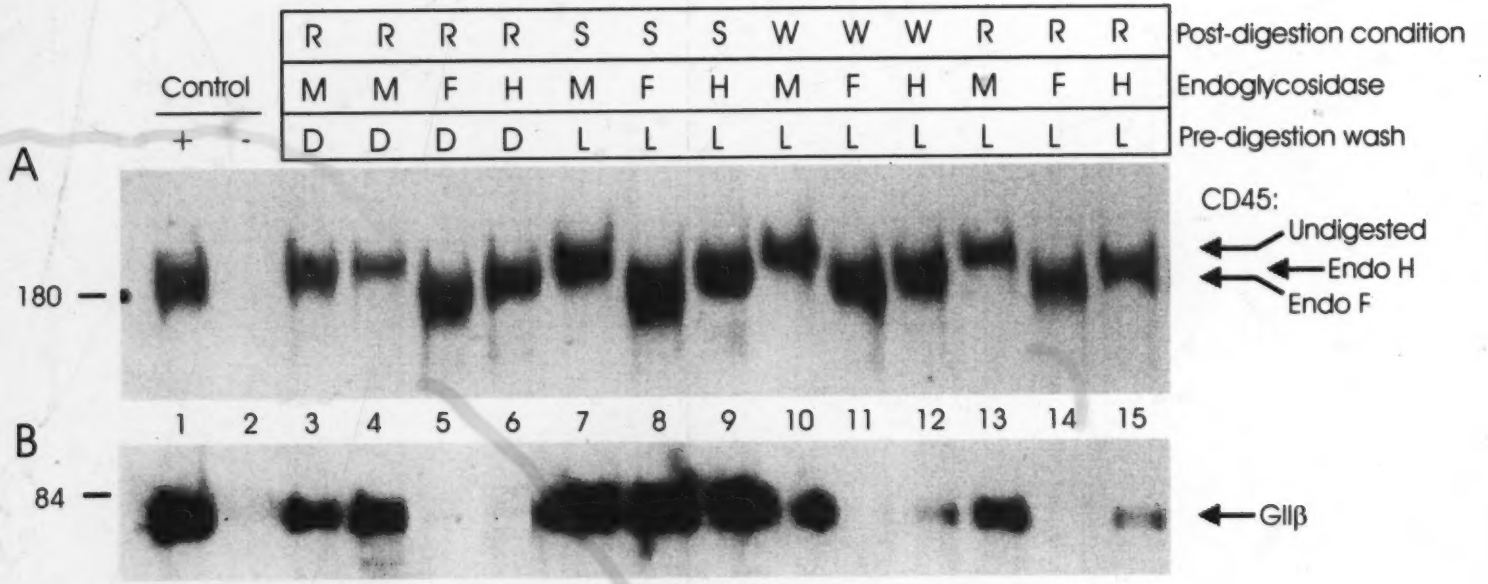
R R R R S S S W W W R R R
Post-digestion condition
Control
M M F H M F H M F H M F H
Endoglycosidase
+ -
D D D D L L L L L L L L L
Pre-digestion wash
A
CD45:
Undigested
Endo H
Endo F
180
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
84
GIIβ

FIGURE 5-10. *N*-linked glycans are required to sustain the association between CD45 and GII$_\beta$, however CD45 exhibits partial resistance to Endo H when glucosidase II is associated. CD45 immunoprecipitates were prepared from lysates of SAKR (*all lanes except 2*) or SAKR/CD45$^-$ cells (*lane 2*). Control immunoprecipitates (*lanes 1-2*) were washed in lysis buffer and boiled immediately in reducing sample buffer. The remaining immune complexes were washed in lysis buffer (*L*) or dissociation buffer (*D*) prior to treatment with Endo F (*F*), Endo H (*H*), or digestion buffer alone as a mock control (*M*). The mock digestions were carried out in Endo F buffering conditions, with the exception of the sample in lane 4, which was incubated in digestion buffer optimized for Endo H. During the overnight digestion, samples were maintained at 37°C with constant rotation. Following the digestion period, one group of samples was boiled in reducing sample buffer (*S*). A second group (*W*) was washed 3 times in lysis buffer before being boiled in sample buffer. The final group (*R*) was washed and incubated with post-nuclear lysates from SAKR/CD45$^-$ cells for the *in vitro* reconstitution of binding assay. Samples were resolved on a 7.5% polyacrylamide gel and transferred to Immobilon-P for consecutive blotting with *A*, anti-95K serum and *B*, anti-80.2 serum.

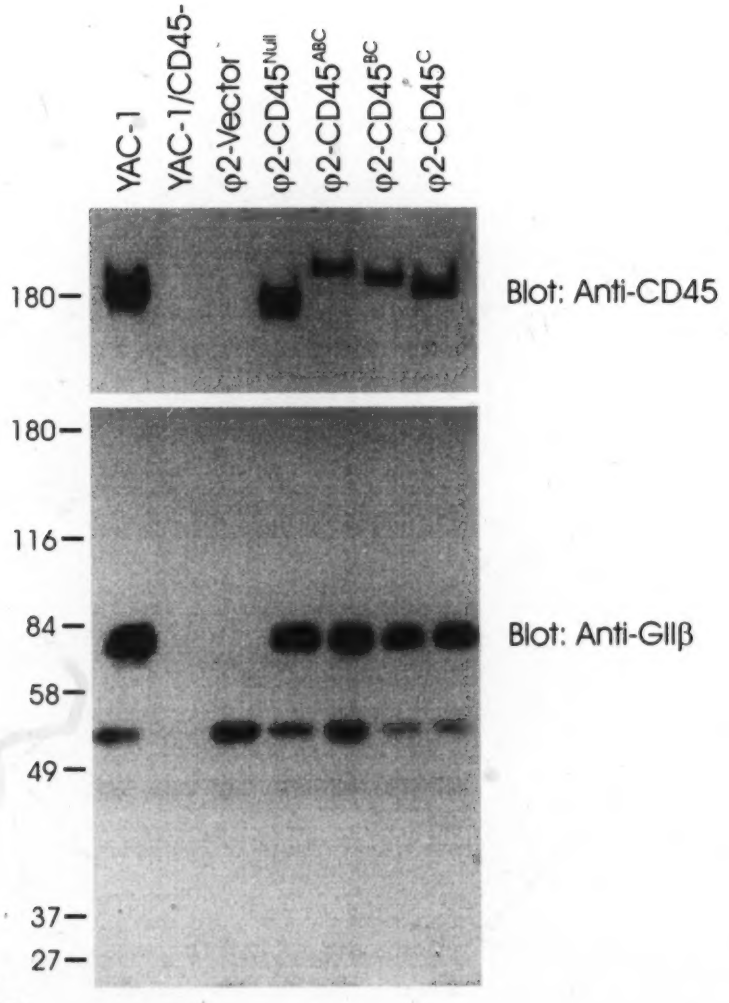

FIGURE 5-11. The β-subunit of glucosidase II is capable of associating with various members of the CD45 isoform family. I3/2 immunoprecipitates were prepared from YAC-1 and YAC-1/CD45⁻ cells. CD45 immunoprecipitates were also prepared from lysates of ψ_2 cells expressing individual CD45 isoforms or, as a control, from cells containing empty vector. The blot was probed with antiserum 95K directed against the cytoplasmic domain of CD45. A parallel experiment was blotted with anti-80.2 serum.

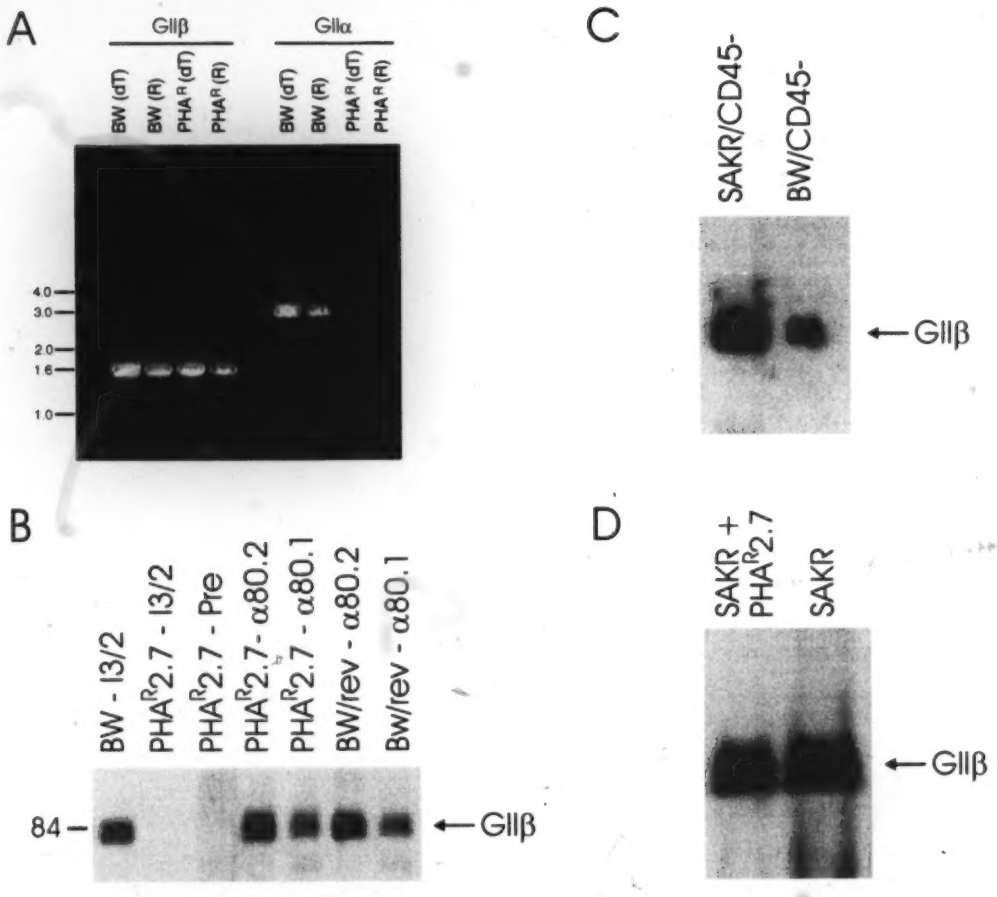

FIGURE 5-12. CD45 from GII$_\alpha$-deficient PHA^{R}2.7 cells cannot associate with endogenous GII$_\beta$ but can associate with GII$_\beta$ from a cell lysate containing wild-type GII$_\alpha$. *A,* RNA extracted from BW and PHA^{R}2.7 was reverse transcribed by random (*R*) or oligo(*dT*) priming. The entire GII$_\alpha$ and GII$_\beta$ ORFs were amplified from template cDNA, resolved on a 0.6% agarose gel, and visualized by staining with ethidium bromide. Standards are indicated in kb. *B,* I3/2 immunoprecipitates were prepared from BW and PHA^{R}2.7. Alternatively, GII$_\beta$ was directly immunoprecipitated from 5 x 10^7 PHA^{R}2.7 cells or 2.5 x 10^7 BW/rev cells with the anti-80.1 or -80.2 sera, or with preimmune serum (*pre*) as a negative control. Blotting was carried out with the 80.1 reagent at 1:5000. *C,* CD45 was immunoprecipitated from PHA^{R}2.7, washed 2 times in dissociation buffer, and incubated with post-nuclear extracts from SAKR/CD45$^-$ or BW/CD45$^-$ cells. The ability of glucosidase II from the lysate to associate with PHA^{R}2.7-derived CD45 was assessed by blotting with anti-80.2 serum. Note that a portion of the beads were lost from the sample that received the BW/CD45$^-$ lysate. *D,* CD45 was immunoprecipitated from a mixed lysate derived by combining extracts from 2.5 x 10^7 SAKR cells and 2.5 x 10^7 PHA^{R}2.7 cells. In parallel, CD45 was immunoprecipitated from 5 x 10^7 SAKR cells. The blot was probed with anti-80.2 serum.

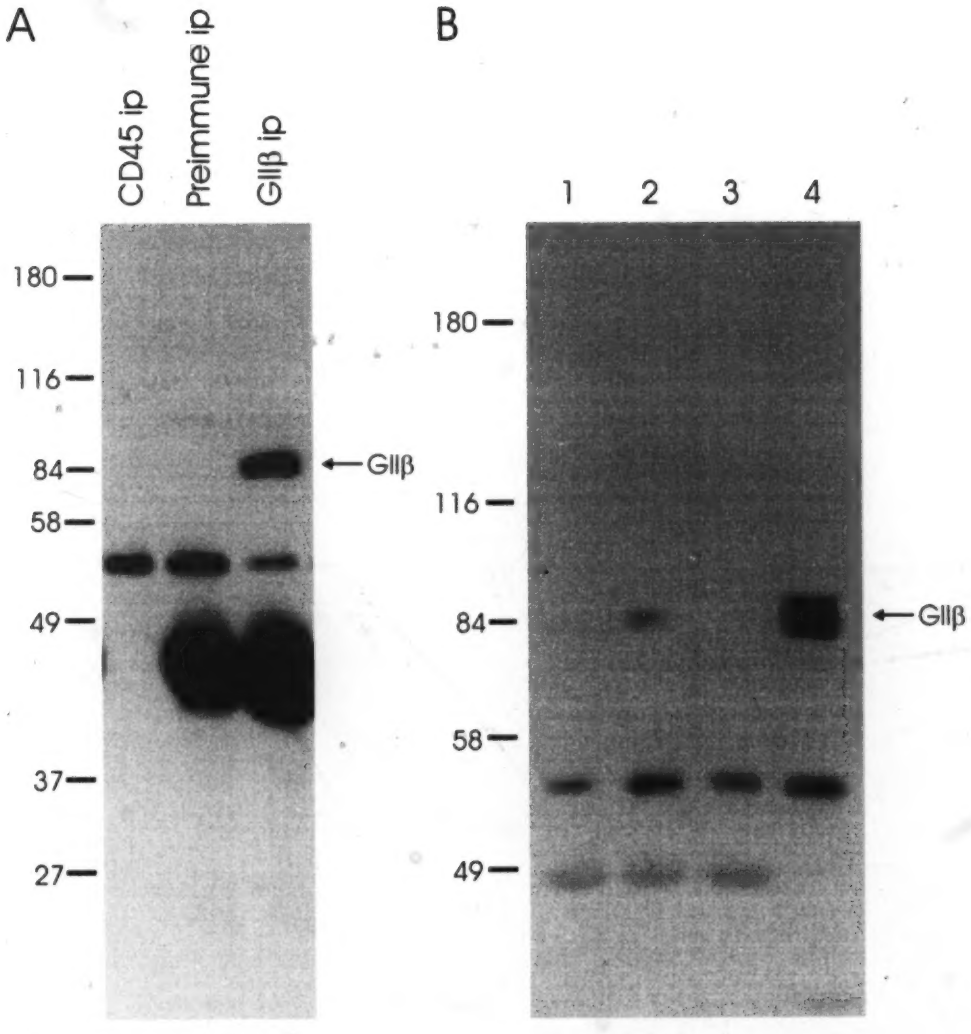

FIGURE 5-13. CD45 and GII$_\beta$ are not associated in CTL clone AB.1, however the association between SAKR-derived CD45 and AB.1-derived GII$_\beta$ can be reconstituted *in vitro*. *A,* CD45 was immunoprecipitated from 1.3 x 10^7 clone AB.1 cells. As a control for expression of GII$_\beta$, immunoprecipitates were prepared in parallel using preimmune serum or 80.2 antiserum. The blot was probed with anti-80.2 serum. *B,* reconstitution of binding assay. CD45 was immunoprecipitated from SAKR cells, washed in dissociation buffer, and mixed with lysis buffer as a negative control (*lane 1*) or lysates from 1.3 x 10^7 clone AB.1 cells (*lane 2*). CD45 was also directly immunoprecipitated from 1.3 x 10^7 clone AB.1 cells and the immune complexes were washed in lysis buffer (*lane 3*). As a positive control, post-nuclear extracts from 10^6 AB.1 cells were loaded onto the same gel (*lane 4*). The blot was probed with anti-80.2 at a dilution of 1:15,000.

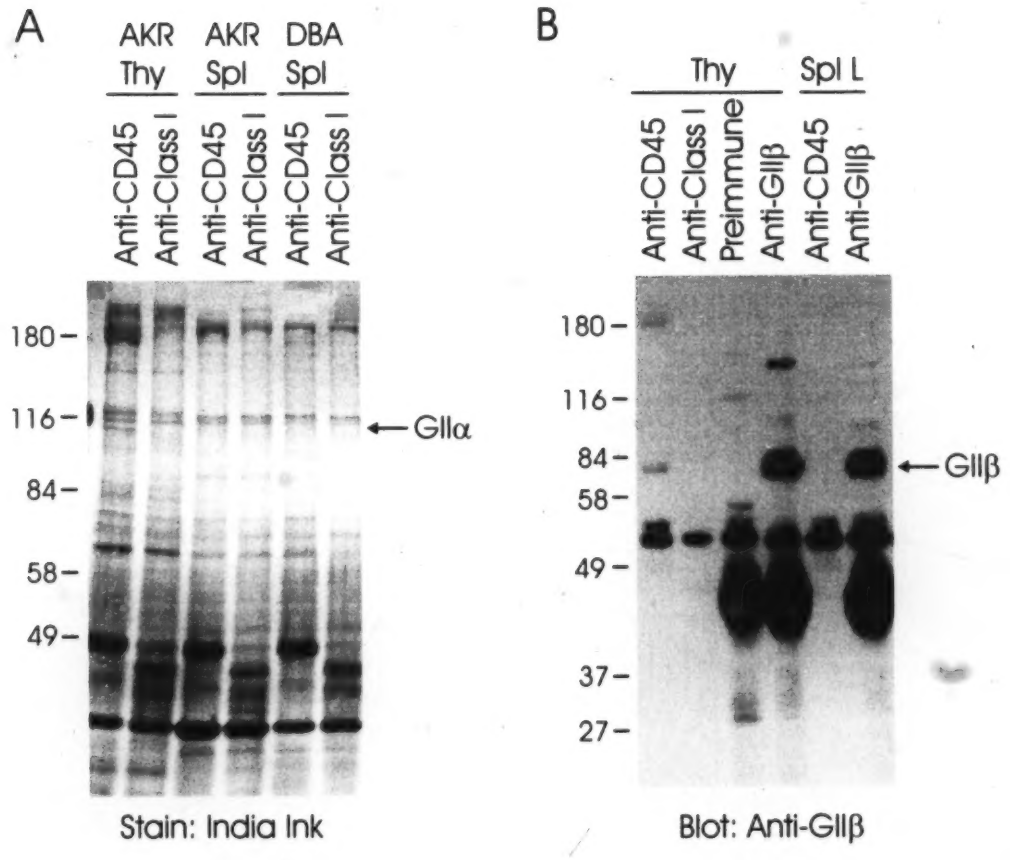

FIGURE 5-14. CD45 is associated with glucosidase II in thymocytes but not splenocytes. *A*, CD45 or, as a control, MHC class I, was immunoprecipitated from lysates of 2.5 x 10^7 thymus (*Thy*) or spleen (*Spl*) cells freshly isolated from young AKR or DBA/2 mice, as indicated. The immunoprecipitates were washed in lysis buffer and proteins were visualized by India ink staining after transfer to Immobilon-P. *B*, thymocytes (*Thy*) and splenic lymphocytes (*Spl L*) isolated from young BALB/c mice were lysed at a density of 4.5 x 10^7/ml for immunoprecipitation under the Ab conditions indicated. The anti-80.2 serum was employed in immunoblot analysis.

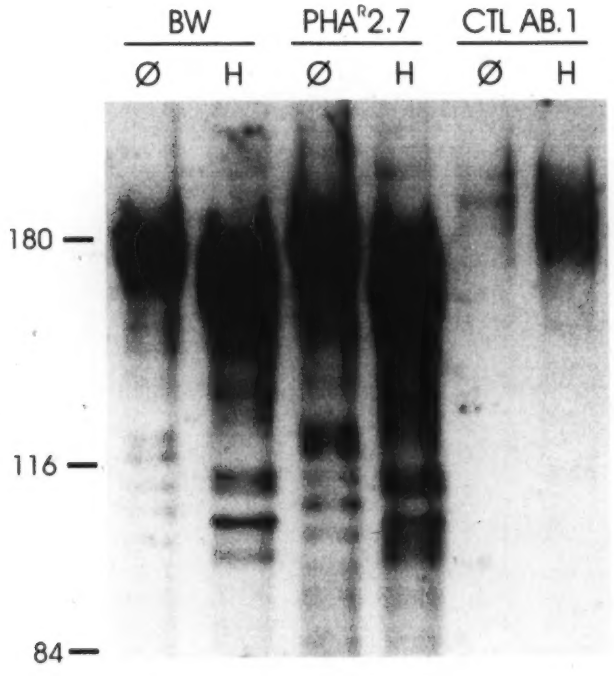

FIGURE 5-15. Endo H-sensitive CD45 can be detected in both immature and mature T-cells. CD45 was immunoprecipitated from 2.5 x 10^7 BW or PHA^R2.7 cells, or from 1.25 x 10^7 clone AB.1 cells. Immune complexes were incubated overnight with Endo H (*H*) or buffer alone (Ø). Samples were resolved on a 7.5% polyacrylamide gel and the blot was probed with anti-95K serum.

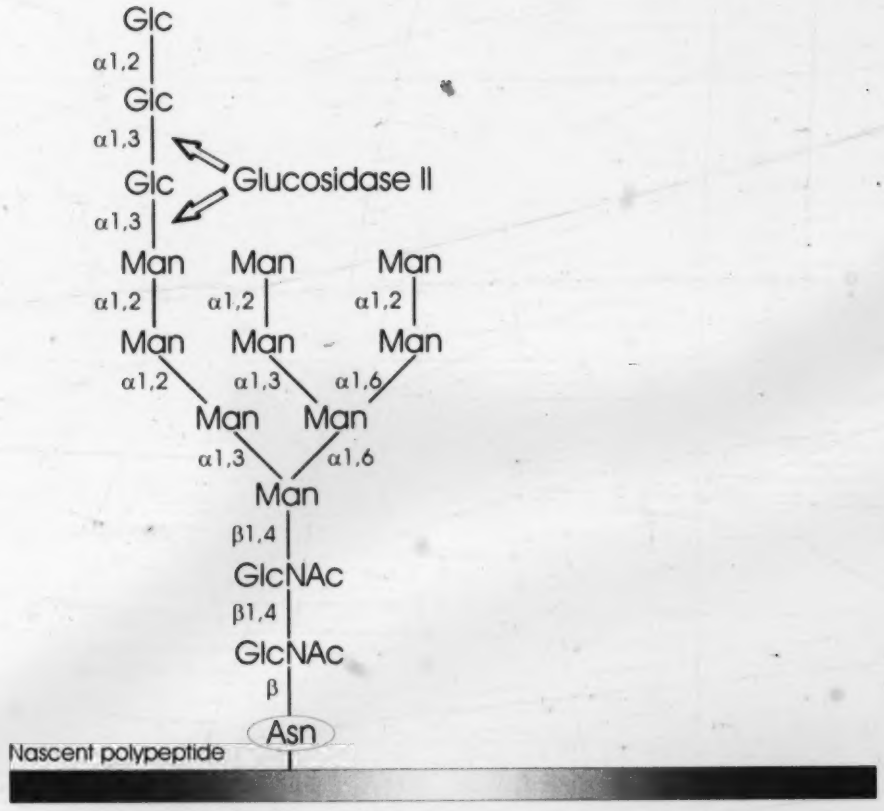

FIGURE 5-16. Structure of the precursor oligosaccharide unit. Shown is the precursor carbohydrate moiety added in the ER to sites of *N*-linked glycosylation conforming to the motif Asn-X-Ser/Thr (where "X" represents any residue except Pro). The two linkages hydrolyzed by glucosidase II (subsequent to cleavage of the outermost α1,2-linked glucose by glucosidase I) are indicated. (Adapted from Gahmberg and Tolvanen, 1996).

CHAPTER VI

ALTERNATIVE SPLICING OF TRANSCRIPTS ENCODING THE α- AND β-SUBUNITS OF MOUSE GLUCOSIDASE II IN T-LYMPHOCYTES

(A version of this chapter is in preparation for submission. Arendt, C. W., Dawicki, W., and Ostergaard, H.L. W. Dawicki carried out PCR amplification and cloning of genomic DNA prepared by C. Arendt. The sequence data obtained by W. Dawicki is presented in Fig. 6-2. All other data in this chapter were obtained independently by C. Arendt.)

A. Introduction

Despite considerable progress in characterizing the catalytic activity and biological functions of glucosidase II, its molecular identity has only recently been elucidated (Trombetta et al., 1996; Arendt and Ostergaard, 1997; Flura et al., 1997). In the process of determining the sequences of transcripts encoding the α- and β-subunits of mouse glucosidase II, multiple cDNA clones were derived by a number of independent approaches (Arendt and Ostergaard, 1997). Closer examination of these clones has revealed that sequence heterogeneity exists at two sites in GII_{α}, designated boxes A1 and A2, and at one site in GII_{β}, termed box B1. The purpose of this study is to investigate whether variable expression of these box segments in T cells can be attributed to a mechanism of alternative mRNA splicing.

B. Results

Heterogeneity in GII_α and GII_β cDNA clones. While cataloguing partial cDNAs encoding the α- and β- subunits of glucosidase II (Arendt and Ostergaard, 1997), three regions were identified that were variably expressed among the panel of clones (Fig. 6-1). The first of these, termed box A1, is located proximal to the amino terminus of the α-subunit of mouse glucosidase II and encodes a segment of 22 amino acids bearing no homology to known sequences. The nucleotide sequence encoding this segment was previously reported in the cloning of full-length mouse GII_α, however cDNA clones have since been identified that lack the box A1 residues. Of five cDNA clones examined, four were missing the box A1 element (Table 6-1). It is hypothesized that a differentially spliced exon segment encodes this 22 amino acid insert. Interestingly, this segment is not present in two independently reported cDNA clones of human GII_α (Nagase et al., 1995; EMBL database clone AJ000332) or in a cDNA clone of pig GII_α (Flura et al., 1997).

The second variable region that has been identified, designated box A2, is located in the α-subunit of glucosidase II at a position 145 amino acids downstream of box A1 (Fig. 6-1). Box A2 encodes a segment of 9 amino acids absent in the previously reported sequence of mouse GII_α. Out of the five mouse GII_α cDNA clones surveyed, one was found to contain the box A2 segment (Table 6-1). Within this small pool of cDNA clones, three isoforms of GII_α could therefore be distinguished: Form 1 (previously reported, box $A1^+$, box $A2^-$), Form 2 (box $A1^-$, box $A2^+$) and Form 3 (box $A1^-$, box $A2^-$). The existence of Form 4 GII_α (box $A1^+$, box $A2^+$) has not yet been confirmed. Like the box A1 segment, box A2 is also absent in both the pig and human cDNA clones of GII_α,

however its variable representation among this panel of mouse cDNA clones suggests that it represents a second site of alternative splicing within the GII_α transcript.

The third and final variable region discovered, box B1, is positioned immediately downstream of a striking acidic stretch near the carboxyl terminus of GII_β (Fig. 6-1). Box B1 codes for a 7 amino acid segment that has been identified in three out of ten mouse GII_β cDNA clones derived by various methods (Table 6-2). Interestingly, an unpublished bovine GII_β cDNA sequence of unknown fidelity (GenBank accession number U49178) contains an element of identical location and similar sequence to the mouse box B1 VQGEQPK segment, although the bovine element (DSQGEQPKD) is slightly longer. The human cDNA sequence reported for a single GII_β clone (Sakai et al., 1989), in contrast, encodes at this position a segment (DSEE) with identity to the first two amino acids in the bovine sequence but no identity to the mouse box B1 element. It is postulated that variable expression of the mouse box B2 element can be ascribed to alternative mRNA splicing, a process that may also account for heterogeneity at the corresponding region of GII_β in other mammals expressing this highly conserved protein.

Genomic sequencing of the regions encompassing boxes A1, A2, and B1. To verify that the three variably expressed exon segments identified in mouse glucosidase II are susceptible to alternative exon splicing, genomic sequence information was sought that would allow exon/intron boundaries to be located and the presence of donor and acceptor splice sites to be confirmed. Genomic DNA was purified from the SAKR T-cell lymphoma cell line from which the cDNA fragments encoding GII_α and GII_β ORFs were isolated (Arendt and Ostergaard, 1997). Oligonucleotide primer pairs flanking boxes A1,

A2, and B1 were employed to amplify these regions of genomic DNA by PCR. PCR reaction products were directly sequenced to exclude the possibility that the variably expressed segments are encoded by two independent alleles differing only at the variable box sites. Fidelity of genomic sequences was further confirmed by parallel cloning of the PCR products and sequencing of plasmid DNA. Consistent with the earlier analysis of GII_α cDNA clones, box A1 was found to be encoded by an internal exon located between a 456-bp 5'-flanking intron (fully sequenced) and a ~0.9-kb 3'-flanking intron (partially sequenced) (Fig. 6-2*A*). The presence of donor and acceptor splice sites at the predicted sites of the intron/exon boundaries supports a model whereby the box A1 exon is either included or excluded from GII_α transcripts by a mechanism of differential mRNA splicing.

Unlike box A1, box A2 is not located adjacent to a 5'-flanking intron, although a ~0.5-kb 3'-flanking intron (partially sequenced) is present (Fig. 6-2*B*). Rather, the first two nucleotides encoding box A2 specify a GT-donor splice site while a second GT-donor splice sequence is present at the 5' boundary of the downstream flanking intron. At the 3' boundary of this same intron is an AG-acceptor splice site, allowing for a splicing reaction that either omits or includes box A2. Together with the cDNA sequence data, this suggests that box A2 comprises a second alternatively spliced exon cassette in GII_α.

Similar to box A2, box B1 in GII_β exists as an exon cassette juxtaposed by a single downstream flanking intron which, in this case, consists of only 77 nucleotides (Fig. 6-2*C*). The pattern of donor and acceptor splice sites mirrors that of box A2, suggesting that a similar mechanism of differential mRNA splicing is operative. In the sequencing of mouse genomic DNA in this region, a 184-nucleotide intron (fully

sequenced) was located between amino acid residues 269 and 270, upstream of box B1 (data not shown). The available cDNA sequence data, however, indicate that splicing at this second intron is not coupled to variable expression of GII_β coding sequences.

PCR analysis of utilization of boxes A1, A2, and B1. The flanking primers used to amplify the regions of genomic DNA encompassing boxes A1, A2, and B1 were also used to examine the representation of these variably expressed segments within reverse-transcribed RNA prepared from various T-cell lines. Amplification of the region containing box A1 generated two DNA fragments corresponding in size to the two different splice forms of GII_α produced by variable inclusion of the 66-bp box A1 segment (Fig. 6-3*A*). Both splice forms could be detected in the transformed T-cell lines SAKR and BW as well as the non-transformed cytotoxic T-lymphocyte clone AB.1. A slight bias favoring amplification of box $A1^-$ DNA fragments was consistently observed under the PCR conditions used. Similar results were obtained regardless of whether the cDNA template was prepared using oligo(dT) or random primers. As presented in Fig. 6-3*A*, these two splice forms of GII_α could also be detected, albeit with lower efficiency, in the $PHA^R2.7$ cell line, a glucosidase II-deficient derivative of the BW thymic lymphoma (Reitman et al., 1982). A titration of the PCR products generated in this reaction revealed that the relative ratios of box $A1^-$ to box $A1^+$ amplicons is roughly similar in BW and $PHA^R2.7$ cells but, unexpectedly, that $PHA^R2.7$ cells express two forms of box $A1^+$ transcripts (Fig. 6-3*B*). The fact that amplification of box $A1^-$ transcripts in $PHA^R2.7$ does not yield a doublet band on this high-resolution gel suggests either that abnormal splicing is occurring during excision of the flanking introns that affects box $A1^+$

transcripts only, or that a small deletion exits within the box A1 coding sequence itself. Whatever the specific nature of the defect, it appears that only one allele is affected, since a band equal in staining intensity to the aberrant fragment is present that comigrates with wild-type box-$A1^+$ amplicons.

Examination of PCR products generated during amplification of the box A2 region allowed differentiation of GII_α isoforms containing or lacking this 27-bp segment (Fig. 6-3*C*). Interestingly, in all cells examined it was observed that amplification of the isoform containing box A2 occurred with considerably greater efficiency than did the isoform lacking this element. In fact, the ability to detect the lower molecular-weight isoform in this assay appeared to be operating near a threshold, as evidenced by inconsistent amplification of the smaller DNA fragment in the two reactions primed from CTL AB.1 cDNA. It is not known whether these differences in isoform amplification by PCR reflect differences in protein expression *in vivo*. Amplification of box $A2^-$ isoforms was not improved by altering the method of reverse transcription priming. The box $A2^-$ cDNA species could not be detected in $PHA^R2.7$ cells; however, this may reflect limitations in assay sensitivity, given the apparent diminution in GII_α transcript levels in this cell (see also Chapter V, Fig. 5-12*A*). Taken together, these data confirm that alternative splicing of the box A2 region can be detected in various T cell populations and raise the possibility that utilization of the downstream donor splice site at the 3' boundary of box A2 (Fig. 6-2*B*) may be favored.

Finally, the PCR assay was used to examine GII_β isoform representation in several T-cell lines. DNA species amplified in this experiment migrated as two closely spaced bands on agarose gels, consistent with there being only a 21-bp difference between

isoforms exhibiting variable inclusion of the box B1 segment (Fig. 6-3*D*). The large and small fragments corresponding to the two splice forms of GII_β were amplified with similar efficiency in all cells examined. Notably, both isoforms could be amplified from $PHA^R2.7$ with an efficiency comparable to that observed in wild-type cells, in agreement with the expression of wild-type levels of GII_β by $PHA^R2.7$ (Chapter V, Fig. 5-12*A*). Interestingly, GII_β protein has been observed to migrate as a doublet on polyacrylamide gels (Arendt and Ostergaard, 1997), a phenomenon that may relate to alternative splicing of the 7 amino acid box B1.

C. Summary of Results

Examination of multiple cDNA clones encoding the α- and β-subunits of mouse glucosidase II, coupled with partial genomic DNA sequencing, has revealed that both subunits are encoded by gene products that undergo alternative splicing in T lymphocytes. The catalytic α-subunit possesses two variably expressed segments, box A1, consisting of 22 amino acids located proximal to the amino terminus, and box A2, composed of 9 amino acids situated between the amino terminus and the putative catalytic site in the central region of the molecule. Box B1, a variably expressed 7 amino acid segment in the β-subunit of glucosidase II is located immediately downstream of an acidic stretch near the carboxyl terminus. Screening of reverse transcribed RNA by polymerase chain reaction confirms the variable inclusion of each of these segments in transcripts obtained from a panel of T-lymphocyte cell lines.

D. Discussion

Possible functions of alternate glucosidase II isoforms. At present, it is only possible to speculate as to the potential biological relevance of the polypeptide regions encoded by the alternatively spliced segments. One possibility is that these regions function to modulate protein-protein interactions, such as those mediating the association between GII_α and GII_β. A variety of empirical approaches indicate that GII_α and GII_β isolated from rat liver microsomes exist as noncovalently linked heterodimers that are highly refractory to dissociation (Trombetta et al., 1996). In the context of the present study, it will be of interest to assess the binding kinetics of each of the four potential isoforms of GII_α with the two different isoforms of GII_β to determine whether the variably expressed segments influence enzyme dimerization.

It is also possible that alternative splicing of glucosidase II influences interactions between the enzyme and its substrates or other resident ER proteins. The functions of glucosidase II closely intersect with those of other ER proteins including glucosidase I, calnexin, calreticulin, and UDP-glucose:glycoprotein glucosyltransferase (Helenius et al., 1997). Interestingly, mouse glucosidase II was initially purified by virtue of its highly stable interaction with the transmembrane protein-tyrosine phosphatase CD45 in immature phenotype T-lymphoma cells such as SAKR and BW, an interaction that does not occur in the mature CTL clone AB.1 (see Chapter V). Given this dichotomy, it is important to note that no obvious qualitative or quantitative differences were observed in terms of isoform preferences between these cell types in the PCR assay. It cannot be formally excluded, however, that subtle differences exist in glucosidase II isoform expression patterns between immature and mature cells that may influence the association

with CD45. Interestingly, microsequence analysis of CD45-associated p116 (GII_α) indicates that it contains the box A1 element (Arendt and Ostergaard, 1997), however it is unclear whether glucosidase II lacking this element is also capable of associating with CD45. It will be important to examine the possible contributions of boxes A1, A2, and B1 to the binding interaction between glucosidase II and CD45 in comparison to other substrates.

Finally, it is necessary to consider that alternative splicing of glucosidase II may alter its intrinsic activity or subcellular localization. Although the putative catalytic site of GII_α is located distal to boxes A1 and A2, nothing is known about how the activity of the enzyme is regulated. In the evolutionarily related lysosomal α-glucosidase, however, alterations in a single amino acid residue located well outside the catalytic site have been shown to ablate enzyme activity (Lin and Shieh, 1995). It is also possible that alternative splicing of glucosidase II might have repercussions on as yet unexamined retention and/or retrieval mechanisms operating to maintain the enzyme in the ER. In this context it is interesting that an isoform of the human invariant chain protein has been identified that lacks an ER retention signal due to alternative initiation of translation (Schutze et al., 1994). While glucosidase II has been shown to localize primarily to the ER in rat and pig hepatocytes (Grinna and Robbins, 1979; Brada and Dubach, 1984; Lucocq et al., 1986), there is also evidence to indicate that in some cell types GII_α (Brada et al., 1990; Arendt and Ostergaard, 1995) and GII_β (Li et al., 1996) are capable of trafficking to the cell surface. Studies focused on the functions of the alternatively spliced domains of glucosidase II may thus reveal additional levels of biological complexity stemming from isoform heterogeneity. Complementary studies in a variety of species will be necessary to

clarify whether the alternatively spliced box sequences, and any isoform-specific functions, are evolutionarily conserved.

Defects in PHA^{R}2.7 GII$_\alpha$ transcripts. The PHA^{R}2.7 mutant, which has been exploited in a variety of experimental systems (Balow et al., 1995; Gabel and Kornfeld, 1982; Kearse et al., 1994; Moore and Spiro, 1992; Ora and Helenius, 1995; Suh et al., 1992; Wiest et al., 1995), has recently been shown to lack GII$_\alpha$ mRNA by Northern blot analysis (Flura et al., 1997). Using highly sensitive PCR-based assays, it has been possible in this study to detect reduced levels of GII$_\alpha$ transcripts in PHA^{R}2.7. Moreover, a unique form of GII$_\alpha$ transcript was identified that appeared to possess an altered box A1 segment. PHA^{R}2.7 GII$_\alpha$ cDNAs have been cloned and very limited sequencing of the 3' and 5' ends has revealed the presence of missense and nonsense mutations (data not shown). It therefore appears that multiple defects exist in PHA^{R}2.7 that prevent the expression of functional glucosidase II (Reitman et al., 1982). These defects may include, in addition to gene-specific mutations, reduced gene transcription, decreased message stability, and/or errors in post-transcriptional processing. Efforts are ongoing to generate Ab reagents to GII$_\alpha$ to address whether mutant protein is expressed in PHA^{R}2.7 that may provide structure-function insights. It will be important to characterize the molecular defects in GII$_\alpha$ in the PHAR2.7 cell line, including the putative box A1 alteration, given that the association between glucosidase II and CD45 does not occur in this cell (see Chapter V).

TABLE 6-1

Summary of box A1 and A2 utilization in GII_{α} cDNA clones

Clone	Size (kb)	Cloning Method	Cell	Box A1	Box A2	Designation
116FL.A	3.1	PCR	SAKR	–	–	Form 3
116FL.B[a]	3.1	PCR	SAKR	+	–	Form 1
116FL.G	3.1	PCR	SAKR	–	–	Form 3
6R5-13	1.6	5' PCR-RACE	SAKR	–	–	Form 3
6R5-14	1.6	5' PCR-RACE	SAKR	–	+	Form 2

[a] The sequence of this cDNA clone has been previously reported (Arendt and Ostergaard, 1997).

TABLE 6-2
Summary of box B1 utilization in GII_β cDNA clones

Clone	Size (kb)	Cloning Method	Cell	Box B1	Designation
80-1	1.5	cDNA library	EL4	–	Form 2
80-2	1.0	cDNA library	EL4	+	Form 1
80-3	1.3	cDNA library	EL4	–	Form 2
80-4	1.5	cDNA library	EL4	+	Form 1
80-5[a]	1.8	cDNA library	EL4	–	Form 2
80FL.1	1.7	PCR	SAKR	+	Form 1
8R3-5	1.5	3' PCR-RACE	SAKR	–	Form 2
8R3-12	1.5	3' PCR-RACE	SAKR	–	Form 2
8R3-17	1.5	3' PCR-RACE	SAKR	–	Form 2

[a] The sequence of this cDNA clone has been previously reported (Arendt and Ostergaard, 1997).

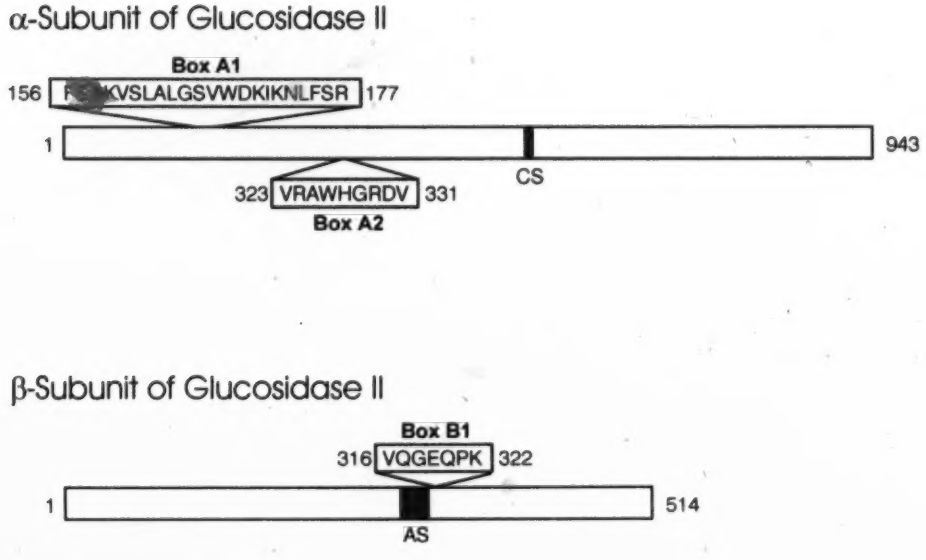

FIGURE 6-1. Distribution of alternatively spliced boxes A1, A2, and B1 in mouse glucosidase II. Schematic representation of the α- and β-subunits of glucosidase II showing the sequences encoded by the variably expressed box A1, A2, and B1 segments. Amino acids presented in standard single-letter code are numbered according to the longest coding sequence for each protein, with position 1 assigned to the first residue following leader peptide cleavage. The putative catalytic site (*CS*) of GII_α and the Glu-rich acidic stretch (*AS*) of GII_β are labeled.

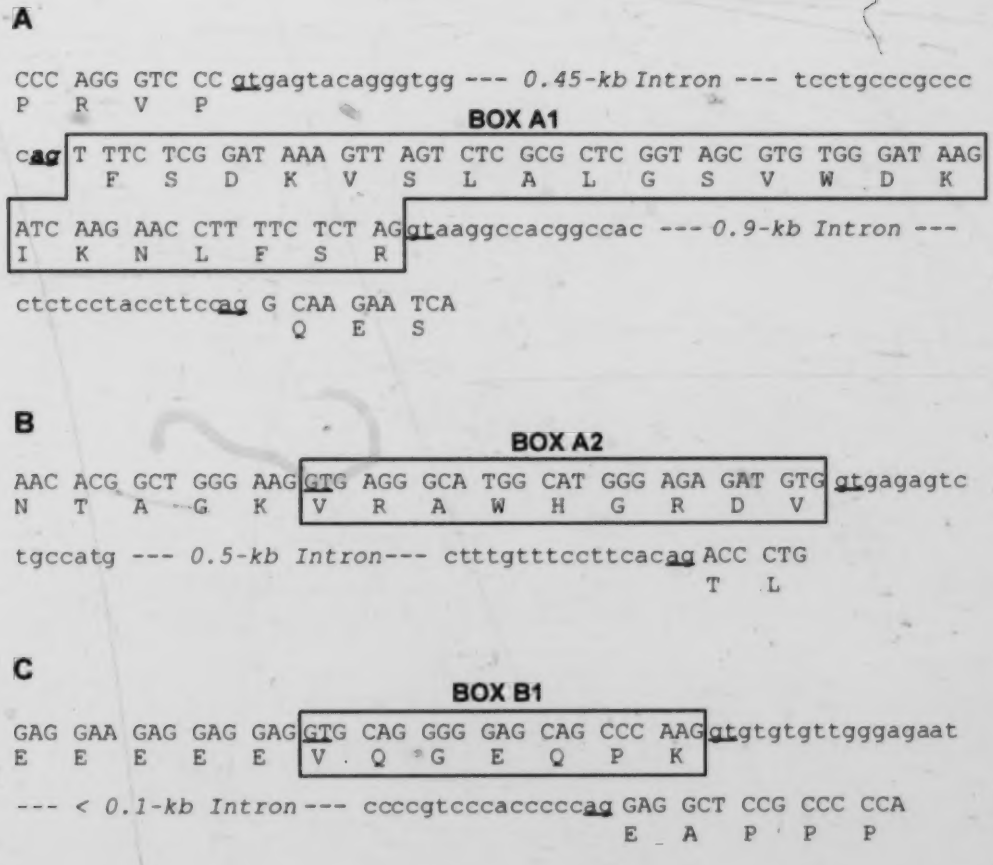
A
CCC AGG GTC CC gtgagtacagggtgg --- 0.45-kb Intron --- tcctgcccgccc
P R V P
BOX A1
cag T TTC TCG GAT AAA GTT AGT CTC GCG CTC GGT AGC GTG TGG GAT AAG
F S D K V S L A L G S V W D K
ATC AAG AAC CTT TTC TCT AG gtaaggccacggccac --- 0.9-kb Intron ---
I K N L F S R
ctctcctaccttccag G CAA GAA TCA
Q E S
B
BOX A2
AAC ACG GCT GGG AAG GTG AGG GCA TGG CAT GGG AGA GAT GTG gtgagagtc
N T A G K V R A W H G R D V
tgccatg --- 0.5-kb Intron--- ctttgtttccttcacag ACC CTG
T L
C
BOX B1
GAG GAA GAG GAG GAG GTG CAG GGG GAG CAG CCC AAG gtgtgtgttgggagaat
E E E E E V Q G E Q P K
--- < 0.1-kb Intron --- ccccgtcccacccccag GAG GCT CCG CCC CCA
E A P P P

FIGURE 6-2. Partial genomic sequences of mouse glucosidase II α- and β-subunits. Mouse genomic DNA flanking the box A1 (*A*), box A2 (*B*), and box B1 (*C*) elements was sequenced to identify donor (*gt*) and acceptor (*ag*) splice sites. Partial intronic sequences are presented in *lowercase* letters, and the approximate sizes of the introns are indicated. Preparation of samples for sequencing was carried out by W. Dawicki.

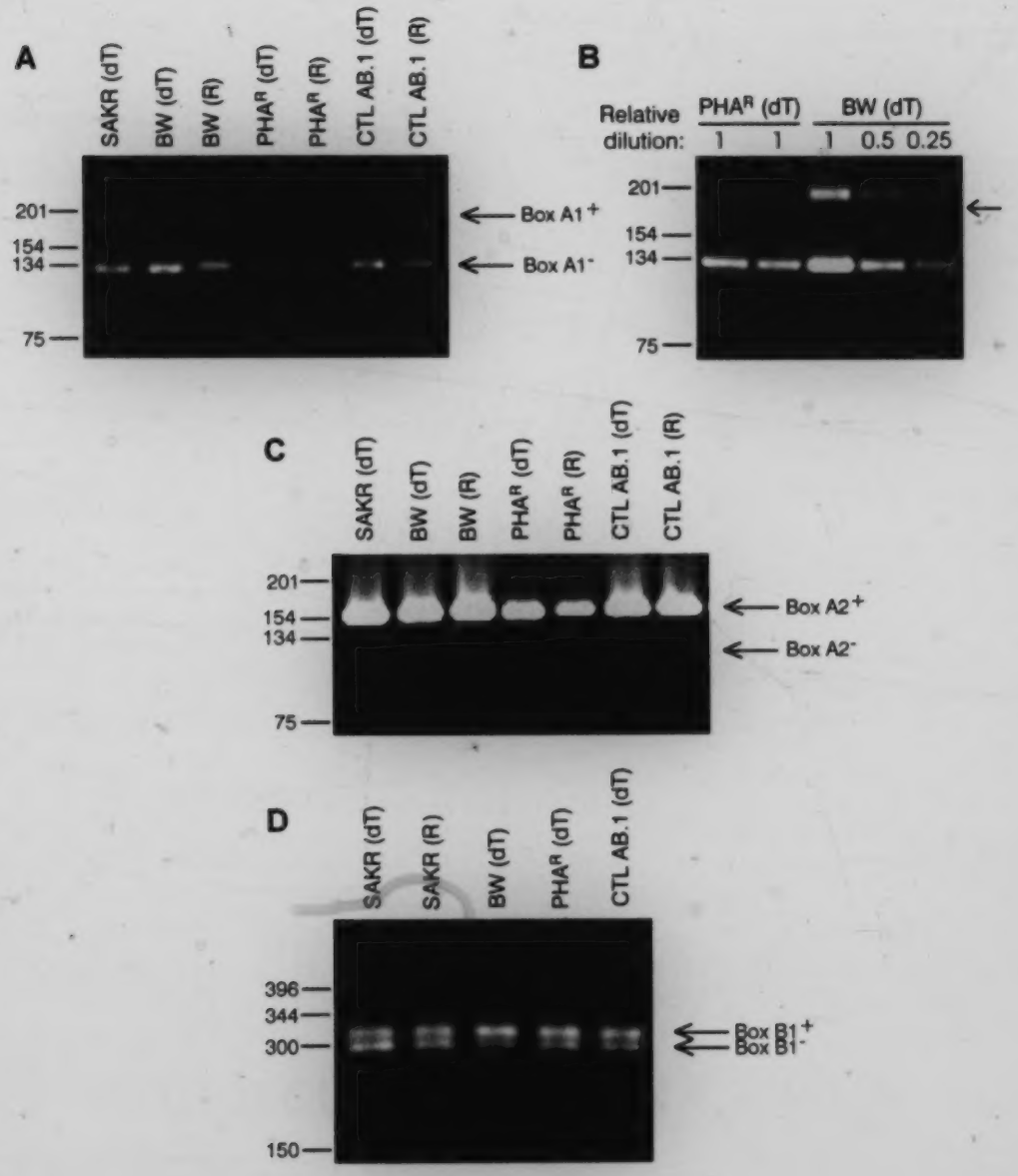

FIGURE 6-3. RT-PCR analysis of isoform distribution. Oligo(*dT*) or random (*R*) primed cDNA prepared from RNA extracted from various T-cell lines was amplified using primers flanking the box A1 (*A & B*), box A2 (*C*), or box B1 (*D*) elements. PCR reaction products were visualized by ethidium bromide staining and sizes of known standards are indicated in bp. In *B*, PCR products from BW have been diluted by the indicated factor relative to those from PHA^{R}2.7. The *arrow* in *B* identifies the unique box A-positive amplicon in reactions primed from PHA^{R}2.7 cDNA.

CHAPTER VII

TWO DISTINCT DOMAINS OF THE β-SUBUNIT OF GLUCOSIDASE II INTERACT WITH THE CATALYTIC α-SUBUNIT

(A version of this chapter has been submitted for publication. Arendt, C. W. and Ostergaard, H.L.)

A. Introduction

The catalytic subunit of glucosidase II is quite unlike most other known processing enzymes in that it lacks a transmembrane segment and a known retention/retrieval motif. However, elucidation of the coding sequence of the associated β-subunit in mouse has revealed the presence of a carboxyl-terminal His-Asp-Glu-Leu (HDEL) sequence (Arendt and Ostergaard, 1997) that is conserved in its highly homologous human counterpart (Sakai et al., 1989), a protein of uncertain function. Since this motif has been shown to be sufficient for recognition by the KDEL receptor (Ozawa and Muramatsu, 1993; Wilson et al., 1993), it is possible that GII_β functions to couple the α-subunit to this retrieval mechanism. Interestingly, GII_β also possesses a putative EF hand domain and a region of negatively charged repeats, features thought to confer Ca^{2+} binding properties to other ER proteins (Booth and Koch, 1989; Sonnichsen et al., 1994; Weis et al., 1994). As there is evidence that for the involvement of a calcium

matrix in the retention of resident ER proteins (Booth and Koch, 1989; Sonnichsen et al., 1994; Weis et al., 1994), it is possible that GII_β invokes multiple mechanisms to ensure localization of GII_α to the ER.

In order to achieve a better understanding of the biological roles of GII_β, molecular analysis of its functional domains is necessary. In the current study, a panel of GII_β fusion proteins has been utilized to identify the regions of GII_β that couple this protein to the catalytic subunit.

B. Results

Two distinct domains of GII_β are capable of associating with α-glucosidase II activity. A schematic of primary sequence features of the β-subunit of mouse glucosidase II is presented in Fig. 7-1. This protein is synthesized with an amino-terminal leader peptide which is absent in the mature form of the polypeptide (Arendt and Ostergaard, 1997). Cys residues are present in at high concentrations near the amino and carboxyl termini, but not in the intervening region. The central region of the molecule contains two putative EF-hands and, nearby, a stretch of Glu repeats flanked by Pro-rich elements. A HDEL tetrapeptide sequence at the carboxyl terminus may interact with the transmembrane "KDEL receptor" (Ozawa and Muramatsu, 1993; Wilson et al., 1993) as a mechanism for recycling glucosidase II to the ER. Indeed, the highly stable interaction between GII_α and GII_β (Trombetta et al., 1996) and the absence of known retention/retrieval motifs in the soluble α-subunit suggest that one function of GII_β may

be to ensure that the catalytic α-subunit is maintained in the ER by coupling it to the KDEL receptor.

Since nothing is known about the sites through which GII_β associates with GII_α, this study was undertaken to map this interaction by making use of a sensitive and quantitative assay for GII_α activity. In this assay, samples are incubated with the chromogenic substrate p-nitrophenyl α-D-glucopyranoside, which is specifically hydrolyzed by glucosidase II at neutral pH (Burns and Touster, 1982; Reitman et al., 1982). In preliminary experiments, this assay was utilized to determine if GII_α activity could be detected in immunoprecipitates of GII_β from mouse SAKR cells, the T-lymphoma line from which the two subunits of glucosidase II were originally purified and cloned (Arendt and Ostergaard, 1997). Immune complexes were captured on Protein A beads, washed, and incubated with the reaction mix. Color change, indicating the presence of GII_α, was quantitated by measuring the optical density of triplicate samples at 405 nm. As illustrated in Fig. 7-2*A*, antisera 80.1 and 80.2, which recognize two distinct regions of GII_β (Fig. 7-1), were capable of coprecipitating the enzymatic activity encoded by GII_α. Preimmune sera from the two rabbits, however, failed to retrieve glucosidase II activity from the cell lysates. The amount of active GII_α coimmunoprecipitated by the 80.1 and 80.2 antisera in this experiment was roughly equivalent to 1% of the activity in the total cell lysate (Fig. 7-2*A*). Titration of SAKR cell lysates indicated that the colorometric assay was within a linear range under these conditions (Fig. 7-2*B*). These data support the notion that the α- and β-subunits of glucosidase II are stably associated and indicate that neither antiserum blocks the ability of these two proteins to interact.

To identify specific regions of GII_β that mediate the association with the catalytic subunit, segments of mouse GII_β spanning the entire length of the molecule were expressed as GST-fusion proteins in *E. coli.* These fusion proteins were incubated with SAKR cell extracts and then captured by addition of glutathione matrix. *In vitro* association of GII_α with the recombinant GII_β proteins was assessed using the colorometric activity assay. The results of this initial binding study are presented in Fig. 7-3. Surprisingly, two non-overlapping constructs, GSTβ(N-258) and GSTβ(273-C), were found to be capable of precipitating GII_α from SAKR lysates. In contrast, no activity was found to associate with GSTβ(112-258), GSTβ(112-344), GSTβ(437-C), or GST alone. It is therefore concluded that two independent portions of the β-subunit of glucosidase II are capable of associating with the catalytic subunit.

Amino acids 1-118 of GII_β specify an amino-terminal binding site for GII_α. To more thoroughly map the amino-terminal binding site of GII_β, a series of progressively truncated versions of the GSTβ(N-258) construct were constructed (Fig. 7-4*A*). As revealed by the binding experiment presented in Fig. 7-4*B*, removal of the first 19, 40, or 59 amino acids of GSTβ(N-258) ablated the association with GII_α. This demonstrates that the amino terminus of GII_β (minus the leader peptide) is essential in mediating the interaction with the catalytic subunit. In contrast, truncating the opposite end of GSTβ(N-258) initially resulted in full retention of binding function. In fact, construct GSTβ(N-118), deleting 140 amino acids at the carboxyl terminus of GSTβ(N-258), displayed robust binding activity. However, truncation of an additional 20 amino acids from this construct resulted in a GST-fusion protein incapable of associating with GII_α, as

did further truncations in this direction. Significantly, interaction domain 1 (ID1), encompassing the region encoded by construct GSTβ(N-118), displays minimal overlap with GSTβ(112-258), the fusion protein against which antiserum 80.1 was generated. This is consistent with the finding that anti-80.1, serum but not construct GSTβ(112-258), precipitates GII_α activity (Fig. 7-2 & Fig. 7-3).

A second binding site for GII_α is contained within amino acids 273-400 of GII_β. The data presented in Fig. 7-3 revealed a second interaction domain (ID2) within GII_β that binds to GII_α. Interestingly, the region encompassed by this domain includes an alternatively spliced 7 amino acid segment (see Chapter VI) that defines two isoforms of GII_β (Fig. 7-1). To address the possible function of this 7 amino acid segment in influencing the association of the α- and β- subunits of glucosidase II, GST-fusion proteins were generated that either contained or lacked this segment (Fig. 7-5*A*). As the results of the binding studies presented in Fig. 7-5*B* demonstrate, this segment does not modulate the association of the two subunits, as detected in this *in vitro* system. However, it is not possible to formally exclude the possibility that this segment influences the interaction of the two subunits *in vivo* in the context of full-length GII_β. The results of Fig. 7-5*B* also show that deletion of 114 carboxyl-terminal amino acids from GSTβ(273-C) to generate construct GSTβ(273-400) preserves the ability to precipitate GII_α activity. However, further truncation of 56 amino acids, yielding construct GSTβ(273-344), ablates this binding activity, consistent with the results obtained with construct GSTβ(112-344) (Fig. 7-3). The finding that 114 residues at the carboxyl terminus of GII_β are excluded from ID2 is in agreement with the ability of antiserum 80.2,

directed against 78 residues at the carboxyl terminus (Fig. 1), to coprecipitate glucosidase II activity (Fig. 2). It is thus concluded that residues 273-400 of GII_β contain a second domain sufficient for associating with the catalytic subunit and that a region within segment 345-400 is necessary for this association.

Glucosidase II activity is dependent upon the presence of the 116-kDa α-subunit. $PHA^R2.7$ is a derivative of the BW thymic lymphoma line that is deficient in glucosidase II activity (Reitman et al., 1982) due to a defect in GII_α expression (Flura et al., 1997). As an additional control for the previous mapping experiments, the parental and mutant cell lines were compared in their abilities to provide GII_α activity in the Ab and fusion protein pull-down assays. The data in Fig. 7-6*A* confirm the specificity of the experimental system employed in this study by demonstrating that GII_α activity cannot be retrieved from the $PHA^R2.7$ cell lysates. To extend this analysis, parallel experiments were carried out to examine the protein constituents of the bead-associated fraction by resolving the precipitated material on polyacrylamide gels and carrying out Coomassie Blue staining. As Fig. 7-6*B* shows, only under conditions that precipitate GII_α activity was a 116-kDa protein precipitated that comigrates with the catalytic subunit of glucosidase II purified from the same cell line. Accordingly, the 116-kDa protein could not be precipitated from $PHA^R2.7$ cells using GSTβ(N-118) or antiserum 80.2 as bait. Significantly, proteins retrieved from BW lysates by GSTβ(N-118) versus GST alone appeared to differ only at the level of the 116-kDa protein, consistent with the notion that GSTβ(N-118) interacts directly with GII_α.

Binding of GII_α to ID1 is not dependent upon the formation of intermolecular disulfide linkages. Interestingly, ID1 spans the entire amino-terminal Cys-rich element of GII_β. Since iodoacetamide irreversibly inactivates the enzymatic activity of glucosidase II (Burns and Touster, 1982), it was not possible to employ the enzyme assay to assess whether the association of GII_α with ID1 requires the formation of disulfide bonds. As an alternative approach, precipitation of GII_α was assessed by Coomassie Blue staining of polyacrylamide gels. As can be seen in Fig. 7-7, pull-down of GII_α by full-length GII_β or GSTβ(N-118) occurs regardless of whether cells are lysed in the presence of 20 mM iodoacetamide, and is only slightly reduced in the presence of the inhibitor (likely a non-specific effect). The conclusion that disulfide bond formation is not required for the association of GII_α and GII_β is consistent with a previous study that conclusively demonstrated the absence of intermolecular linkages between the two subunits of glucosidase II (Trombetta et al., 1996).

Endogenous GII_β does not associate with GST-GII_β fusion proteins. Although previous biochemical data indicate that the α- and β-subunits of glucosidase II interact as noncovalently-linked heterodimers (Trombetta et al., 1996), it is possible that glucosidase II may be capable of forming higher order multimers *in vivo*, as suggested in some of the earlier literature (Burns and Touster, 1982; Brada and Dubach, 1984). By extension to the present mapping studies, it is possible that ID1 and/or ID2 represent homooligomerization domains through which the GII_β GST-fusion proteins are capable of associating with additional molecules of full-length GII_β present in the cell extracts, thereby indirectly linking the recombinant proteins to the catalytic subunit of glucosidase

II. To address this issue, proteins precipitated from SAKR cells using GST-fusion proteins encoding ID1 or ID2 were subjected to Western blot analysis using specific antiserum to GII_β. As shown in Fig. 7-8, this sensitive methodology failed to reveal the presence of T-cell derived full-length GII_β in the bead fraction. This is in contrast to the readily detectable levels of full-length GII_β precipitated by anti-80.1 serum, a reagent that reproducibly precipitates less GII_α activity than GSTβ(N-138) under these conditions (data not shown). Taken together, these results fail to provide evidence for homooligomerization of GII_β and support a model whereby ID1 and ID2 interact directly with the catalytic subunit.

C. Summary of Results

Insights have been sought into the functional domains of GII_β through the definition of regions of the molecule that interact with the catalytic subunit of glucosidase II. A panel of recombinant β-subunit GST-fusion proteins has been screened for the ability to precipitate glucosidase II activity, revealing the existence of two non-overlapping interaction domains (ID1 and ID2) that are individually capable of associating with GII_α. ID1 encompasses 118 amino acids at the amino terminus of the mature polypeptide, terminating well upstream of the putative EF-hand domain of GII_β. Although ID1 spans the entire amino-terminal Cys-rich element of GII_β, disulfide linkages are not required for its association with GII_α. ID2, located near the carboxyl terminus, is contained within amino acids 273-400, a region occupied in part by a stretch of acidic residues but not by the carboxyl terminal Cys-rich element. Variable usage of 7

alternatively spliced amino acids within a 56 amino acid region of ID2 possessing a critical binding determinant does not influence the *in vitro* association of the two subunits.

D. Discussion

Biological functions of the two subunits of glucosidase II. Biochemical characterization of glucosidase II has indicated that it is unique from most other ER and Golgi processing enzymes that are single-subunit integral membrane proteins in that it exists as a complex of two non-covalently-linked soluble subunits (Trombetta et al., 1996). The α-subunit, comprising a 116-kDa protein in mouse, possesses a catalytic consensus sequence (Arendt and Ostergaard, 1997) and appears to represent the functional enzymatic unit of the complex, based on a gene-disruption experiment in *S. cerevisiae* (Trombetta et al., 1996). The β-subunit, an alternatively-spliced protein (see Chapter VI) that migrates as a doublet at approximately 80-kDa on polyacrylamide gels, contains a number of potentially interesting primary sequence features, including a putative EF-hand domain, an acidic stretch, Pro- and Cys-rich elements, and a carboxyl-terminal HDEL tetrapeptide (Arendt and Ostergaard, 1997). The function of the β-subunit is at present unclear. However, since GII_{α} lacks any known motifs for ER retention or retrieval, one possibility is that the β-subunit may serve as a link to the retrieval mechanism provided by the KDEL receptor. In this context, it is intriguing that the HDEL tetrapeptide, which presumably needs to remain accessible to the KDEL receptor, does not reside within ID2. The prediction arising from the present study,

therefore, is that the binding conformation adopted when GII_β associates with GII_α allows the HDEL motif to freely protrude into the lumenal space.

Model for the physical interaction of GII_α with GII_β. That the present experiments implicate not one but two domains of GII_β in the interaction with GII_α is surprising, but consistent with biochemical evidence demonstrating the high associative stability of the two glucosidase II subunits (Trombetta et al., 1996). Analysis of purified glucosidase II by sucrose gradient fractionation, gel filtration, and non-denaturing polyacrylamide electrophoresis suggests that the two subunits exist as a 1:1 heterodimeric complex (Trombetta et al., 1996). Together, the available data suggest a model whereby ID1 and ID2 on a single molecule of GII_β, while individually capable of associating with the α-subunit, synergize in their binding interaction with a single molecule of GII_α. While no evidence has been obtained to indicate that ID1 or ID2 encode homooligomerization domains *per se*, glucosidase II heterodimers may closely interact with one another and other ER proteins as part of a larger protein network (Tatu and Helenius, 1997). It will be of interest to elucidate the relative pool sizes and stability of monomeric versus heterodimeric GII_α/GII_β within the ER when Ab reagents to the α-subunit become available.

The conclusions derived from this study are based upon observations with multiple GST-fusion proteins encompassing ID1 and ID2 and two antisera directed at other portions of the molecule. As with all such studies employing recombinantly expressed proteins, a degree of caution is required in interpreting the results. Future mutagenesis studies will be useful in unraveling the relative contributions of ID1 and ID2

to the binding of the α-subunit and in identifying critical residues. Since glucosidase II is a soluble enzyme, it should be possible to derive crystals for structural analysis of molecular interactions occurring between the α- and β-subunits. It will be particularly interesting to determine whether ID1 and ID2 are juxtaposed in the native structure of GII_β to form a contiguous binding interface that might interact with a single domain of GII_α. Interestingly, ID1 superimposes the Cys-rich element at the amino terminus of GII_β, while ID2 is located adjacent to the carboxyl-terminal Cys-rich element. Reducing versus non-reducing gel analysis of purified glucosidase II has shown that intramolecular disulfide linkages are present in the β-subunit (Trombetta et al., 1996). It will be important to determine whether such linkages serve to bring the amino and carboxyl termini of GII_β into close apposition.

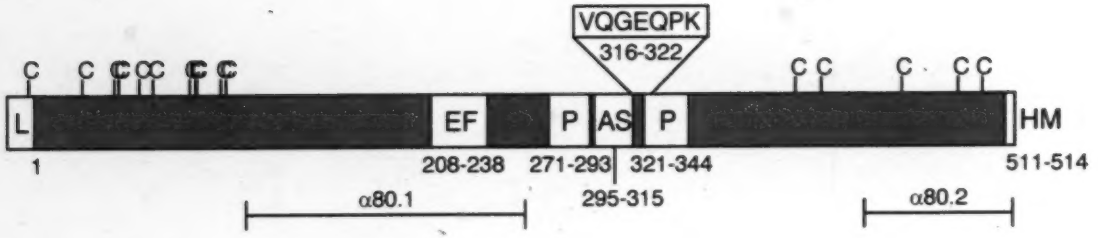

FIGURE 7-1. Schematic diagram of GII_β. Shown are the locations of the leader peptide (*L*), putative EF-hand domain (*EF*), pro-rich elements (*P*), acidic stretch (*AS*), and HDEL motif (*HM*) of GII_β. The distribution of individual Cys residues (*C*) is also indicated. Amino acids are numbered starting with the first residue of the mature protein and include the alternatively spliced box B1 element (residues 316-322). Regions to which antisera 80.1 (α*80.1*) and 80.2 (α*80.2*) were derived are indicated.

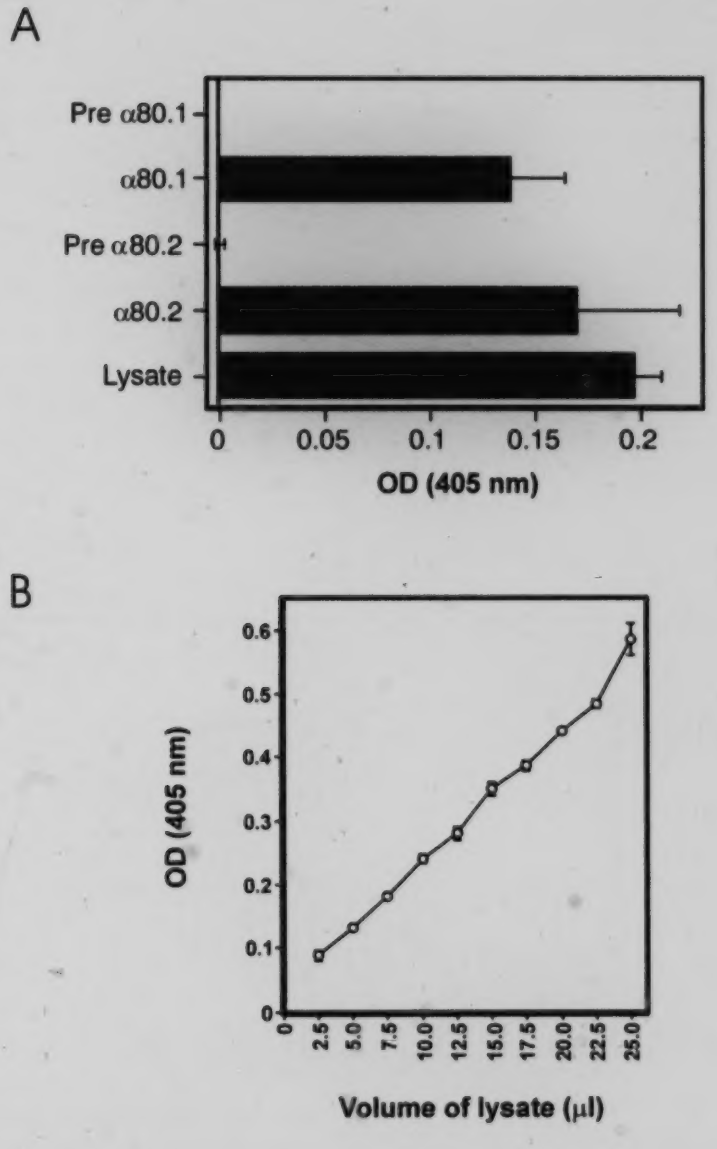

FIGURE 7-2. Precipitation of glucosidase II activity by GII$_\beta$ antisera. *A*, post-nuclear extracts from 5 x 10^7 SAKR cells were incubated for 30 min with 5 μl of 80.1 or 80.2 antiserum, or preimmune serum from the corresponding rabbits (*Pre*). Antisera were captured by addition of protein A beads for 90 min, and immune complexes were washed and subjected to p-nitrophenyl α-D-glucopyranoside assay. Glucosidase II activity was also measured in post-nuclear extracts from 5 x 10^5 cells (*Lysate*). OD_{405} values are expressed as the mean of three independent assays for each condition, with standard error values depicted. *B*, titration of SAKR cell lysates, assayed as described in *A*.

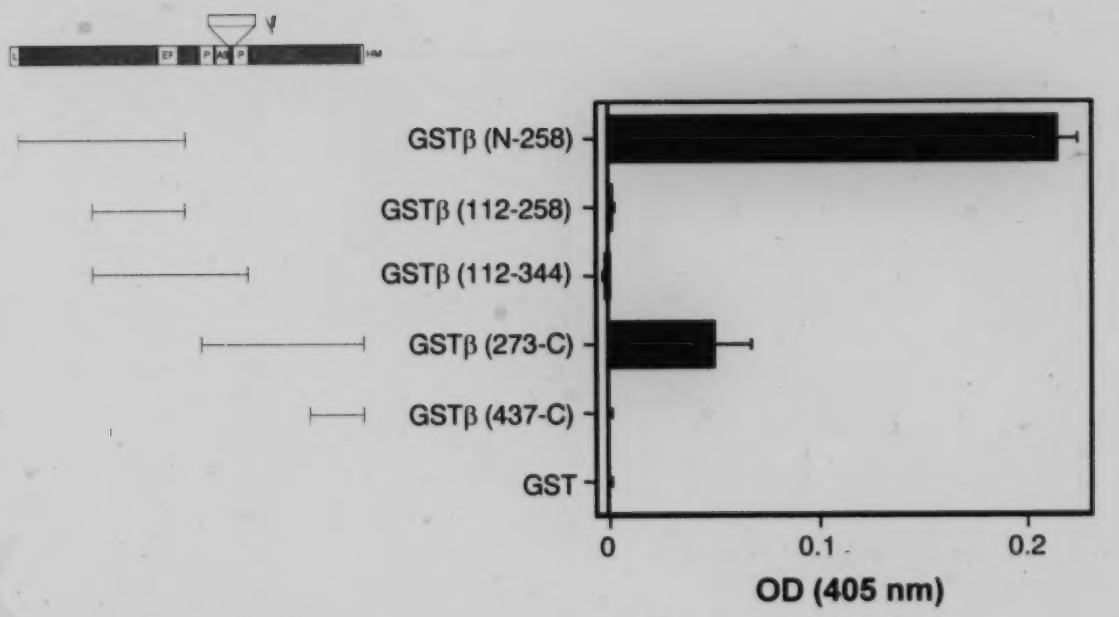

FIGURE 7-3. Precipitation of glucosidase II activity by GII_β GST-fusion proteins. A panel of recombinant GII_β GST-fusion proteins was assayed for the ability to precipitate glucosidase II from lysates of 5×10^7 SAKR cells. Immobilized glutathione was added to lysates preincubated with 10 μg of each fusion protein, and pull-down of glucosidase II activity was quantitated by p-nitrophenyl α-D-glucopyranoside assay. Amino acid designations are given in parentheses, where *N* indicates the first amino acid of the mature protein and *C* signifies carboxyl-terminal amino acid 514.

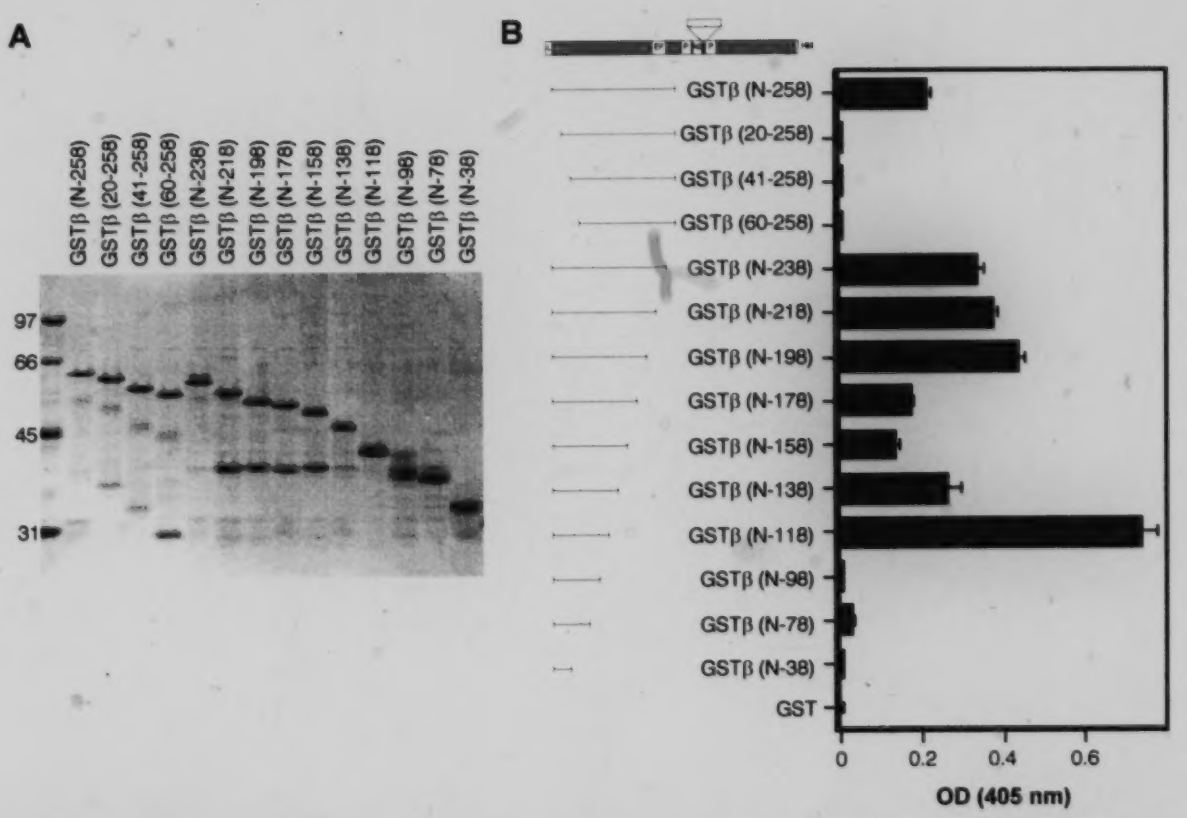

FIGURE 7-4. Mapping of interaction domain 1 in GII$_\beta$. GST fusion proteins corresponding to various truncations of recombinant protein GSTβ(N-258) were purified from *E. coli* lysates. *A*, Coomassie Blue-stained 10% polyacrylamide gel showing 1 μg of each fusion protein compared to 1 μg of various standard proteins. *B*, each construct was assayed for pull-down of glucosidase II activity from SAKR cell lysates as detailed in the legend to Fig. 7-3.

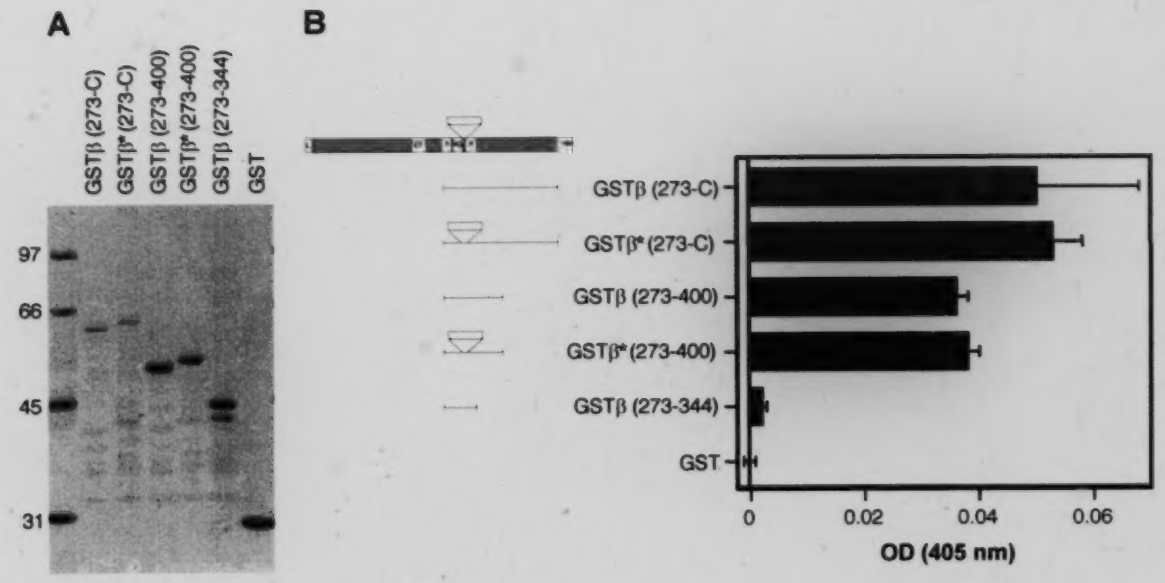

FIGURE 7-5. Mapping of interaction domain 2 in GII$_\beta$. GST-fusion proteins were generated to various regions within 273-C that either contained (indicated with an *asterisk*) or lacked the 7 amino acid variably expressed segment within this interaction domain. *A*, 1 μg each of the various fusion proteins, along with an equivalent amount of standard proteins, were resolved by SDS-PAGE and stained by Coomassie Blue dye. *B*, graphical depiction of glucosidase II activity precipitated by each of the fusion proteins from SAKR cell lysates as measured by p-nitrophenyl α-D-glucopyranoside assay.

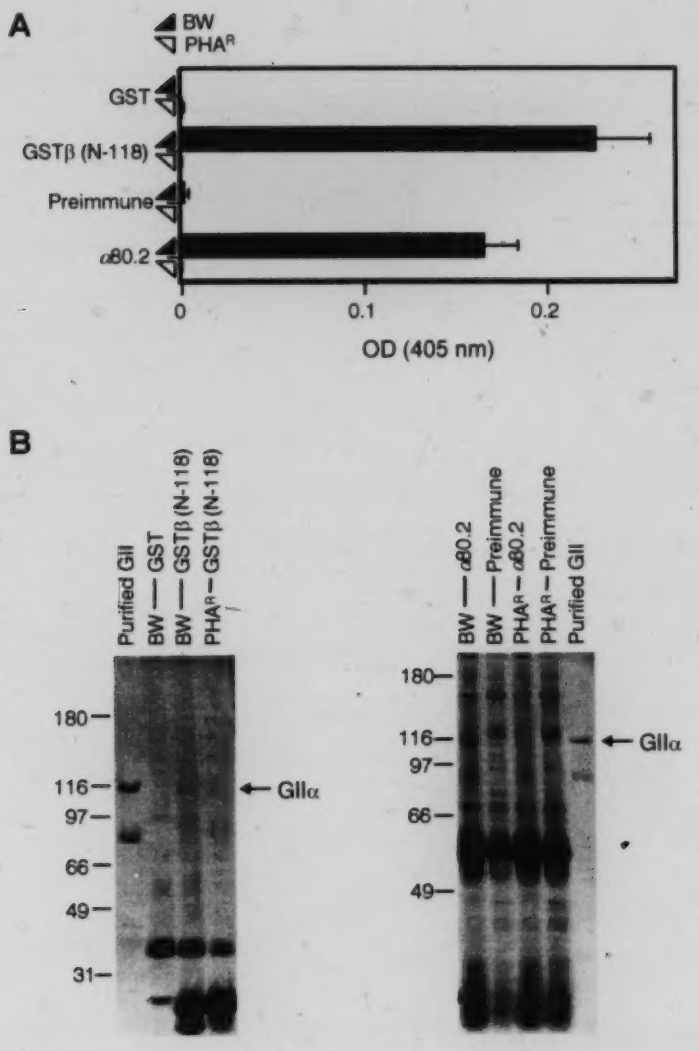

FIGURE 7-6. A 116-kDa protein cannot be precipitated from PHA^{R}2.7 lysates by GII$_\beta$ antibodies or fusion proteins. *A*, lysates from 5 x 10^7 BW or PHA^{R}2.7 cells were incubated with anti-80.2 serum, preimmune serum, GSTβ(N-118) or GST alone for 30 min. Antibodies or fusion proteins were recovered by addition of protein A or glutathione beads, respectively, and glucosidase II activity was assessed as usual by colorometric assay. *B*, proteins recovered in parallel experiments to those described in (*A*) were separated on polyacrylamide gels and subjected to Coomassie Blue staining. To allow for comparison of GII$_\alpha$ gel mobility, purified glucosidase II obtained previously (Arendt and Ostergaard, 1997) was also resolved on these gels.

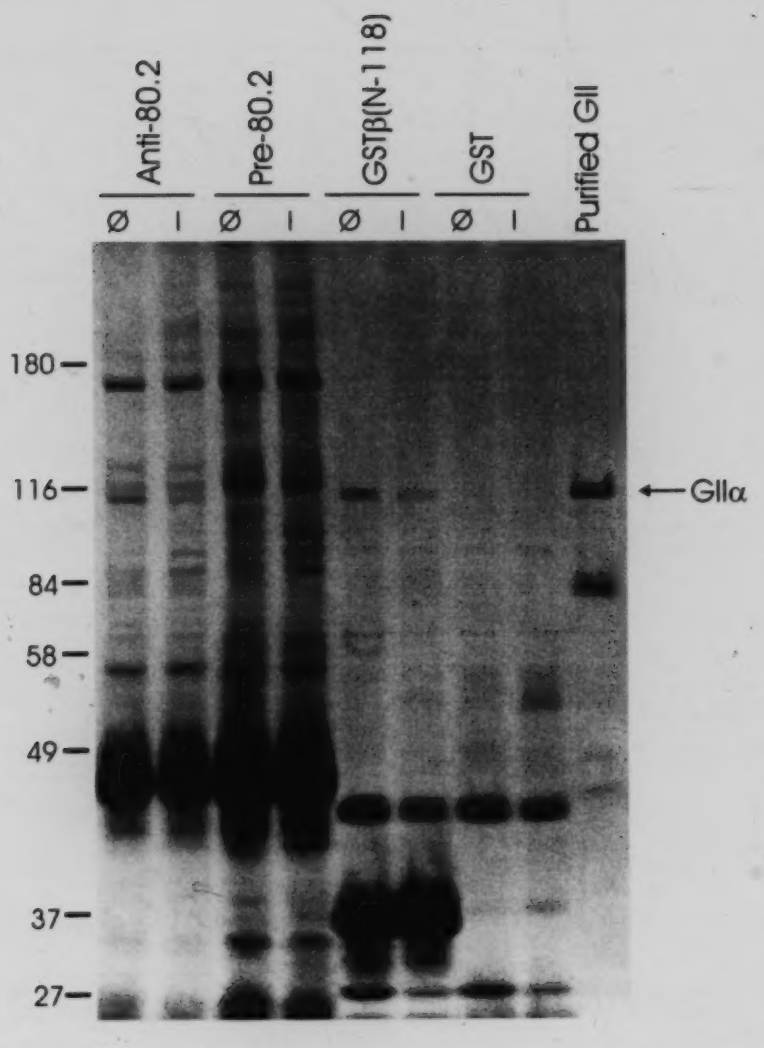

FIGURE 7-7. The association between glucosidase II α- and β-subunits is not dependent upon disulfide bond formation. SAKR cells were lysed in the presence (I) or absence (Ø) of 20 mM iodoacetamide. Pull-down experiments were performed using the indicated Ab reagents or recombinant proteins. Material remaining associated with the bead fraction after 3 washes in lysis buffer was examined by Coomassie Blue staining. Purified glucosidase II prepared from SAKR cells was loaded alongside these samples.

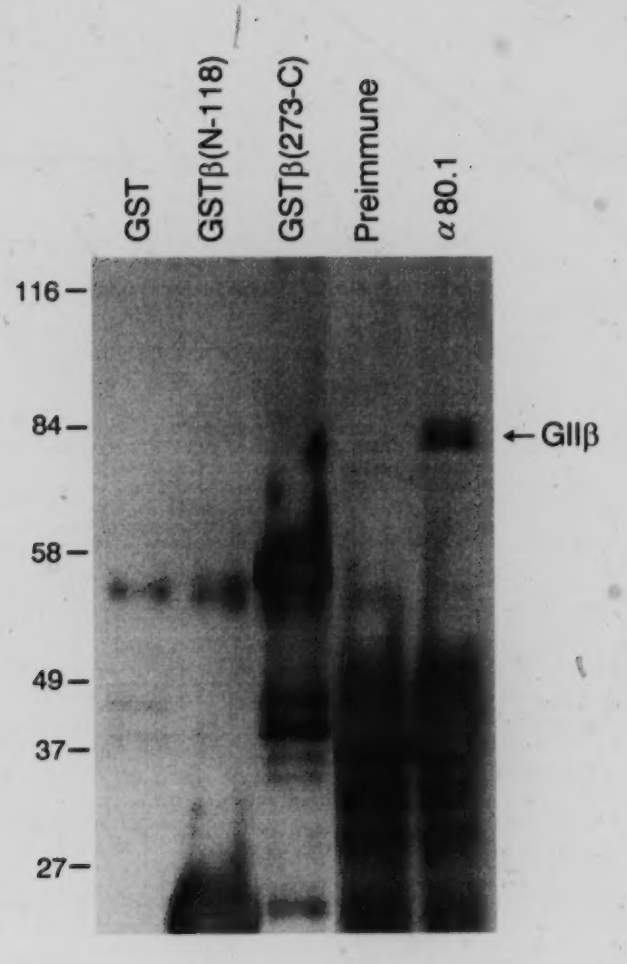

FIGURE 7-8. GII_β GST-fusion proteins encoding ID1 and ID2 fail to associate with full-length GII_β. Lysates from 5 x 10^7 SAKR cells were subjected to immunoprecipitation with 5 µl of either preimmune (*pre*) or anti-80.1 serum. Alternatively, GST pull-downs were carried out by addition of 10 µg GSTβ(N-118), GSTβ(273-C), or GST alone. Precipitated proteins were resolved on a 7.5% polyacrylamide gel, transferred to Immobilon-P, and detected with anti-80.2 serum.

CHAPTER VIII

GENERAL DISCUSSION

A. Functions of Glucosidase II in Glycoprotein Biogenesis

Glycosylation of transmembrane and secreted proteins is a multi-step process that begins in the ER and continues as proteins transit the Golgi apparatus. Each vesicular compartment along this pathway contains specialized enzymes that catalyze the removal or addition of carbohydrate residues. In the ER, *N*-glycosylation is initiated by *en bloc* transfer of a triantennary precursor oligosaccharide moiety containing a tri-Glc extension to a nascent polypeptide (Liu et al., 1979). Cleavage of the terminal Glc residue is catalyzed by glucosidase I, a type II membrane protein of the ER (Kalz-Fuller et al., 1995). The soluble enzyme glucosidase II is responsible for hydrolyzing the two remaining Glc units. Kinetic studies reveal that removal of all three Glc residues proceeds rapidly, with the first two being cleaved cotranslationally (Hubbard and Robbins, 1979). Interestingly, the process of Glc removal appears to be coupled to egress of proteins from the ER (Helenius, 1994). Since Glc modification of glycoproteins does not occur as proteins traverse the Golgi compartments, the relevance of this seemingly redundant yet highly conserved pathway of Glc addition followed by removal occurring in the ER was not immediately apparent.

More recently, a number of elegant studies have revealed an intimate link between Glc processing events in the ER and the coordination of protein folding and assembly (Fig. 8-1). It is clear from these studies that cleavage of the outermost α1,3-linked Glc by glucosidase II is essential for allowing nascent polypeptides to interact with the membrane-bound ER resident calnexin and its luminal homologue calreticulin (Hammond et al., 1994; Hebert et al., 1995; Ora and Helenius, 1995; Wada et al., 1997). Calnexin and calreticulin are members of a new family of lectin proteins with specificity for monoglucosylated precursor oligosaccharides (Peterson et al., 1995; Ware et al., 1995; Spiro et al., 1996; Zapun et al., 1997; Vassilakos et al., 1998). The interaction of polypeptides bearing monoglucosylated carbohydrates with calnexin/calreticulin initiates a cycle of protein folding that is distinct from that provided by classical chaperones in that its initiation is solely dependent on the glycosylation status, rather than the folding status, of the substrate protein (Hammond et al., 1994; Rodan et al., 1996; Zapun et al., 1997). This pathway is also distinct from those governed by classical chaperones in that it is the processing of Glc, rather than the hydrolysis of ATP, that facilitates the multiple cycles of folding that are necessary for most nascent proteins to achieve their native conformations (Ganan et al., 1991; Helenius, 1997). Upon completion of the first folding cycle, glucosidase II cleaves the innermost α1,3-linked Glc, generating a non-glucosylated oligosaccharide product that transiently dissociates from calnexin/calreticulin (Hebert et al., 1995; Hebert et al., 1996; Rodan et al., 1996; Wada et al., 1997). Non-glucosylated glycoproteins that have not yet achieved their proper conformations are recognized by UDP-Glc:glycoprotein glucosyltransferase (UGGT), which adds back a single α1,3-linked Glc (Sousa et al., 1992), allowing for another cycle

of calnexin/calreticulin-assisted folding (Hebert et al., 1995; Hebert et al., 1996; Wada et al., 1997). Once the final conformation is achieved, the glycoprotein is recognized by glucosidase II but not UGGT (Sousa and Parodi, 1995; Zapun et al., 1997), permitting its egress from the ER. Thus, a multi-protein "quality control apparatus" exists in the ER in which UGGT acts as a folding sensor, calnexin/calreticulin function as lectins with chaperone-like properties, and glucosidase II serves as the "engine" responsible for driving the initiation and propagation of folding cycles. In support of the significance of glucosidase II in this cyclic model of protein folding, inhibition of glucosidase II activity after a single cycle of folding has been shown to inhibit protein folding *in vitro* (Hebert et al., 1996; Wada et al., 1997).

The importance of glucosidase II in functioning at the nexus of oligosaccharide processing and protein "quality control" is further supported by a number of *in vivo* studies in which glucosidase II activity has been ablated by mutagenesis, as in the case of the BW-derived $PHA^R2.7$ thymic lymphoma line (Reitman et al., 1982), or by treatment with chemical inhibitors such as castanospermine, bromoconduritol, or 1-deoxynojirimycin. Glycoproteins from cells deficient in glucosidase II activity, in addition to exhibiting decreased levels of complex-type oligosaccharides (Datema et al., 1982; Reitman et al., 1982), are unable to associate with calnexin/calreticulin (Hammond et al., 1994; Kearse et al., 1994; Hebert et al., 1995; Ora and Helenius, 1995). This undermining of the quality control apparatus of the ER is manifested in reduced rates of protein folding (Tector and Salter, 1995), accelerated protein degradation (Kearse et al., 1994), reduced expression of cell-surface proteins (Trowbridge and Hyman, 1978; Arakaki et al., 1987; Edwards et al., 1989; Balow et al., 1995), and defects in protein

secretion (Lodish and Kong, 1984; Yeo et al., 1989). Interestingly, in most cases these effects are observed for specific subgroups of proteins but not others, and no major repercussions on cell proliferation or viability have been reported. The lack of a more severe phenotype in glucosidase II deficient cells may be attributed to activation of alternative chaperone and processing pathways (Moore and Spiro, 1992; Balow et al., 1995; Parlati et al., 1995).

B. Molecular Identity of Glucosidase II

The biochemical activity of mammalian glucosidase II has been extensively characterized since its identification in microsome fractions two decades ago (Ugalde et al., 1978; Grinna and Robbins, 1979; Ugalde et al., 1979; Michael and Kornfeld, 1980; Ugalde et al., 1980; Burns and Touster, 1982; Brada and Dubach, 1984; Hino and Rothman, 1985). Until recently, however, confusion has persisted as to the true molecular identity of this enzymatic activity. In an effort to rectify this situation, Trombetta et al. (1996) purified glucosidase II from rat microsomes by sequential chromatography on DEAE cellulose, Concanavalin A-Sepharose, and Mono Q. Comprehensive biochemical analysis of the purified material revealed that glucosidase II is unique from other ER and Golgi processing enzymes in two aspects. First, it is composed of two strongly associated protein subunits (GII_α and GII_β) that appear to assemble as a non-covalently linked heterodimer. Second, despite the ability of glucosidase II to partition to membrane fractions (Grinna and Robbins, 1979; Ugalde et al., 1979; Michael and Kornfeld, 1980; Brada and Dubach, 1984), it is completely soluble

in the absence of detergents. Extensive amino-terminal and internal microsequence information was obtained on GII_{α} and GII_{β}, leading to the identification of homologous human cDNA clones in the sequence databases.

Contemporaneous to this work, it was discovered that GII_{α} and GII_{β} coimmunoprecipitate with the highly glycosylated transmembrane PTP CD45 in a variety of T-cell lines (Arendt and Ostergaard, 1997). Cloning of the mouse cDNAs and subsequent sequencing of genomic DNA revealed that GII_{α} contains two regions of alternative exon splicing, while GII_{β} contains one such region. Moreover, analysis of the coding regions of these two proteins revealed that both are highly conserved between a variety of mammalian species and contain a number of putative functional domains. GII_{α} was found to share an eight amino acid catalytic consensus sequence with Family 31 glucosyl hydrolases and to possess extensive homologies in this putative catalytic domain with glucosidase II homologues recently identified in wide range of eukaryotic species. In accordance with these observations, the glucosidase II-deficient $PHA^{R}2.7$ cell line was found to exhibit a significant reduction in GII_{α} transcripts, as revealed by PCR analysis, a deficiency that appeared even more severe in the Northern blot analysis of Flura et al. (1997). That GII_{α} represents the enzymatically active component of the glucosidase II heterodimer has been confirmed by a gene ablation experiment in *Saccharomyces cerevisiae*, an organism which, interestingly, does not encode a GII_{β} homologue within its genome (Trombetta et al., 1996). The identification of mouse cDNAs encoding GII_{α} paves the way for structure-function analysis of the putative catalytic site and the alternatively spliced segments, possibly in the context of reconstitution-type experiments employing the $PHA^{R}2.7$ cell line.

Sequence analysis of GII_β also revealed a number of potentially important functional domains. Two potential regions of Ca^{2+} binding are present: a striking negatively charged stretch composed of Glu repeats, and a domain encompassing two putative EF hands. If capable of coordinating Ca^{2+}, these motifs may function as retention domains by maintaining the protein in contact with the Ca^{2+}-rich matrix of the ER lumen (Booth and Koch, 1989; Sonnichsen et al., 1994), a phenomenon that may involve the formation of loose contacts with other ER proteins (Tatu and Helenius, 1997). Since GST-fusion proteins encompassing each of these putative Ca^{2+} binding domains have been generated, Ca^{2+} overlay assays can be performed to verify their function and to explore the intriguing possibility that alternative splicing of the box B1 region, which juxtaposes the Glu-rich region, impinges on Ca^{2+} binding activity.

Another region of GII_β that bears hallmarks of a functional domain is the carboxyl-terminal HDEL sequence. This sequence is likely to be recognized by the KDEL receptor, which accumulates in an ill-defined "salvage compartment" extending at least as far as the *cis* Golgi and functions to retrieve resident ER proteins from the secretory pathway (Vaux et al., 1990; Weis et al., 1994). The KDEL receptor was so named because it was initially thought to do so by interacting exclusively with carboxyl-terminal sequences conforming to the Lys-Asp-Glu-Leu motif. More recently, however, it has been shown that the KDEL receptor binds with greater affinity to HDEL-containing proteins than to those bearing KDEL motifs (Wilson et al., 1993). If the HDEL motif in GII_β indeed serves a retrieval function (which awaits verification by mutagenesis), then it may provide a functional explanation for the association of GII_β with GII_α, the latter of which possesses no identifiable ER retention or retrieval motifs. In this context, it is of

interest that biochemical mapping experiments have allowed identification of two non-overlapping domains in GII_β that interact with GII_α, and that the carboxyl-terminal HDEL sequence is not a constituent of these domains. This finding is consistent with the postulated requirement for this tail sequence to remain accessible to the KDEL receptor. If the regions of GII_α responsible for the association with GII_β can be mapped, GII_α mutants that cannot bind GII_β could be expressed in $PHA^R2.7$ to elucidate whether GII_β is solely responsible for the ER localization of GII_α.

C. Subcellular Localization of Glucosidase II

The development of rabbit antisera recognizing pig glucosidase II (Brada and Dubach, 1984) permitted the investigation of its subcellular localization. Immunoelectron microscopy revealed that in pig hepatocytes glucosidase II is distributed in the rough ER, smooth ER, and in transitional elements between the ER and Golgi apparatus (Lucocq et al., 1986). Labeling of Golgi cisternae was not observed. In striking contrast to these results, the same group observed that glucosidase II is found primarily in post-Golgi structures, including vesicles underlying the plasma membrane, in pig kidney tubular cells (Brada et al., 1990). Moreover, a subset of pig kidney GII_α was found to possess Endo H-resistant linkages and sialic acid modifications. Burns and Touster (1982) have also obtained evidence for complex glycosylations on glucosidase II, consistent with the protein transiting Golgi compartments. Interestingly, calreticulin, another soluble "resident" ER protein that possesses a retrieval sequence recognized by the KDEL receptor and a Ca^{2+}-binding domain conferring high efficiency ER retention

(Sonnichsen et al., 1994), has paradoxically been found to associate with a Golgi mannosidase (Spiro et al., 1996) and to acquire complex glycosylations characteristic of the *trans* Golgi (Michalak et al., 1992). Together, these studies contravene the dogmatic notion that resident ER proteins are incapable of trafficking beyond the *cis* Golgi. Interestingly, in SAKR cells it is possible to obtain weak labeling of a protein that possesses the one-dimensional electrophoretic mobility characteristics of GII_α, suggesting that at least a small proportion of this protein transits to the cell surface. Now that Ab reagents to GII_β are available, it will be important to clarify whether this protein remains stably associated with GII_α under conditions where ER retention and retrieval are usurped. The existence of as many as 6 heterodimeric forms of glucosidase II generated by different combinations of GII_α and GII_β isoforms (and possibly more, if other alternatively spliced segments exist) may impinge on the trafficking characteristics of the enzyme complex.

Adding further complexity to the issue of glucosidase II subcellular localization are a number of seemingly contradictory reports on GII_β, referred to as 80K-H in the earlier literature. Two groups have identified this protein in soluble and membrane fractions and argued that it is present in the cytoplasm. The first group carried out the cDNA cloning of 80K-H, claiming that the protein product was a highly serine phosphorylated PKC substrate in human squamous carcinoma cells (Sakai et al., 1989), although they later reported that the product of the cDNA clone was a poor substrate for this kinase (Hirai and Shimizu, 1990). The second group employed a blotting reagent to 80K-H and argued that it is a major PTK substrate in mouse and human fibroblasts that functions downstream of the fibroblast growth factor receptor (Goh et al., 1996).

Paradoxically, though, phosphorylation of less than 1% of the total cellular pool of this protein was observed, and the findings of this study have been challenged by the discovery of a protein that better corresponds to the target of this search (Kouhara et al., 1997). In a similar fashion, early biochemical characterization of CD45-associated p116 (GII_α) indicated that it too is tyrosine phosphorylated (Arendt and Ostergaard, 1995). Given what is now known about the molecular identities of GII_α and GII_β, in particular the fact that both contain cleavable signal sequences, it would seem unlikely that these proteins exist in the cytoplasm. A more reasonable explanation for the results obtained in these studies is that the breakdown of cellular compartmentalization occurring upon detergent lysis permitted the low-level, non-specific phosphorylation of glucosidase II by cellular kinases.

More recently, data have been obtained suggesting that 80K-H (GII_β) is expressed at the surface of cells of the monocyte-macrophage lineage where it serves as one component of a receptor for proteins modified by advanced glycation endproducts (AGEs) (Li et al., 1996b). AGEs are a heterogeneous series of complex adducts resulting from the non-enzymatic reaction of free-amino groups of proteins or phospholipids with reducing sugars such as Glc, a reaction that is accelerated under conditions of hyperglycemia (Vlassara, 1997). AGE-modified proteins are cable of forming covalent crosslinks and are implicated in a number of pathologies, including atherosclerosis and Alzheimer's disease (Stitt et al., 1997; Yan et al., 1996). A number of receptor families have been identified that are capable of mediating the uptake of AGE-modified proteins, in turn eliciting cytokine production (Stitt et al., 1997). Intriguingly, the pool of GII_β implicated in functioning as a component of an AGE receptor complex was copurified

with OST-48, an ER protein that functions in the transfer of precursor oligosaccharide moieties to acceptor Asn residues (Li et al., 1996b). The relevance of the absence of GII_α in this purified receptor complex is difficult to interpret, since samples were subjected to heat denaturation prior to chromatographic separation, which may have permitted dissociation of this component. Flow cytometric analysis and immunohistochemical staining of non-permeabilized cells have been performed to examine the surface distribution of GII_β, revealing its presence on peripheral blood mononuclear cells, astroglia, endothelial cells, smooth muscle cells, and monocytes/macrophages (Li et al., 1996b; Stitt et al., 1997). While these studies provide additional evidence that GII_β is not exclusively an ER resident protein and provocatively suggest that GII_β may perform multiple cellular functions, the conclusions are weakened by omission of a number of crucial controls. For example, the AGE-binding studies were performed on enriched plasma membrane preparations that were not evaluated for contamination by ER-derived membranes (Li et al., 1996b). Further, AGE binding by purified GII_β could not be demonstrated and the sole evidence for its "coreceptor" function derives from the ability of GII_β-directed antiserum to partially inhibit AGE binding to purified membranes (Li et al., 1996b). Thus, while the results of the cell staining studies are intriguing, the ability of GII_β to contribute to AGE binding requires further substantiation.

D. Association of Glucosidase II with CD45

As a processing enzyme of the ER, glucosidase II interacts in a rapid and transient fashion with presumably all precursor oligosaccharides bearing α1,3-linked Glc

extensions. Complete and permanent removal of α1,3-linked Glc by glucosidase II occurs rapidly for most proteins, and is coupled to termination of the process of chaperone-assisted folding (Helenius, 1994). The finding that CD45, but not other glycoproteins, is capable of remaining stably associated with glucosidase II raises the provocative possibility that the off-rate governing the interaction of glucosidase II with CD45 is much lower than it is for other substrates of the processing enzyme. Although the structural requirements for glucosidase II binding to CD45 have not been fully elucidated, Endo H-sensitive linkages on CD45 are absolutely required for initiation of the association *in vitro*. However, in CTL clone AB.1, CD45 possesses Endo H-sensitive oligosaccharides in the steady state and yet a stable interaction with glucosidase II does not occur. Interestingly, this defect is not absolute in that AB.1-derived glucosidase II is capable of stably associating with CD45 from more immature cells *in vitro*. These surprising findings indicate that dramatic differences exist in the composition of Endo H-sensitive glycans on CD45 between different cell types and/or that other undefined but cell type-specific factors regulate its association with glucosidase II. Interestingly, the mature-type cells in which the association is not detected generally express higher molecular-weight CD45 isoforms than immature-type cells, in which the association does occur (Trowbridge, 1978). However, this factor alone does not seem to dictate whether glucosidase II can stably bind CD45, since glucosidase II is capable of associating with low, intermediate, and high molecular-weight forms of CD45 expressed in fibroblasts. Furthermore, the dichotomy between immature- and mature-type cells is not absolute, in that GII_{α} apparently interacts with CD45 in the IL-2-dependent but Ag-independent CTL clone 2C.

Cell type-specific controls that may act upon CD45 and dictate its ability to stably associate with glucosidase II include factors influencing its conformational state and/or glucosylation status. The tertiary structure of CD45 may be altered by a variety of post-translational modifications, including glycosylation, disulfide bond formation, and phosphorylation. A recent study of class I molecules has revealed that the spatial distribution of sites of *N*-linked glycosylation influences associations with folding chaperones and modulates outcomes of protein folding (Zhang and Salter, 1998). Extending these observations to CD45, it is possible that cell type-specific controls regulating which sites of CD45 *N*-glycosylation are utilized may influence its protein structure. In terms of glucosylation, it is possible that dramatic differences exist in the steady-state Glc composition of CD45 in different cell types. Since glucosidase II recognizes α1,3-linked Glc, the glucosylation status of CD45 may be the sole factor governing its ability to associate with glucosidase II in detergent lysates. If this is so, the biological mechanisms by which large pools of glucosylated CD45 are maintained in certain cells (e.g. SAKR) but not others (e.g. clone AB.1) will require elucidation to clarify if the regulation of glucosidase II differs in these cell types. A variety of factors such as sequestration, competition, transit rates, and the protein/ionic composition of the ER microenvironment may restrict the ability of glucosidase II to recognize CD45 as a substrate *in vivo* in some cell types but not others. Exploration of these issues may yield new biological insights into cell type-specific mechanisms governing the maturation of complex glycoproteins such as CD45.

Assuming that the catalytic domain of GII_{α} mediates the initial binding of glucosidase II to CD45, it is extremely puzzling as to why this enzyme should stably

interact with a substrate. In the monoglucosylated state, CD45 would be expected to be more stably associated (*in vitro* or *in vivo*) with calnexin/calreticulin, which effectively compete with glucosidase II for monoglucosylated substrates (Zapun et al, 1997). The reconstitution of binding studies indicate that CD45 can undergo multiple cycles of binding to glucosidase II *in vitro*, confirming that glucosidase II does not rapidly deplete CD45 of oligosaccharide structures necessary for initiation of binding. Though counterintuitive, it is necessary to consider that glucosidase II might bind CD45 in a α1,3-Glc-dependent manner but not efficiently catalyze the deglucosylation reaction. It is possible that CD45 may negatively modulate the enzymatic activity of glucosidase II, perhaps through protein-protein interactions that have yet to be confirmed, but which are known to mediate the interaction of calnexin with certain proteins (Margolese et al., 1993; Ware et al., 1995; Wiest et al., 1995). Alternatively, protein-protein interactions may stabilize the association of these proteins following an initial oligosaccharide-dependent binding step. To begin to unravel these issues, it will be necessary to fully characterize the glucosylation status of CD45 incubated in the presence and absence of glucosidase II. It will also be important to confirm whether CD45 immunoprecipitated from AB.1 cells or splenocytes is devoid of α1,3-linked Glc.

E. Functional Consequences of the Interaction between CD45 and Glucosidase II

Since glucosidase II localizes primarily to the ER, it is assumed that the initial binding to CD45 occurs in this compartment. One intriguing question that has not been addressed in this study is whether the interaction of glucosidase II with the glycosylated

ectodomain of CD45 modulates the functions of this PTP. It remains unresolved as to whether the cytoplasmic domain of CD45 performs a function during transit of the protein to the cell surface. However, provocative data have been obtained that CD45 qualitatively and quantitatively regulates Ca^{2+} release from intracellular stores (Volarevic et al., 1992), regulation which may be exerted by CD45 acting at the level of intracellular membranes (Shivnan et al., 1996). Intriguingly, tyrosine phosphatase inhibitors have been found to block vesicular transport in endocrine cells, although the mechanisms by which this occurs have not been elucidated (Austin and Shields, 1996). The stable interaction of glucosidase II with CD45 *in vitro*, if occurring *in vivo*, has the potential to exert a direct, ligand-like regulatory influence on the activity of CD45. In addition, if glucosidase II is capable of influencing the trafficking of CD45, it may regulate the PTP by influencing its subcellular distribution. Two PTPs (PTP-1B and $STEP_{61}$) have been described that are sequestered in ER membranes and are activated by proteolytic cleavage, resulting in redistribution to the cytoplasm (Frangioni et al., 1992; Frangioni et al., 1993; Bult et al., 1996). Several investigators have detected large intracellular pools of CD45 in neutrophils (Lacal et al., 1988), T-hybridoma (Minami et al., 1991), and T-leukemia cells (Shivnan et al., 1996). In one study, the intracellular pool size was estimated at 30% of total cellular CD45 and was found to accumulate in a Golgi compartment (Minami et al., 1991). Remarkably, these intracellular pools of CD45 are capable of redistributing to the cell surface (Lacal et al., 1988) or undefined destinations (Minami et al., 1991; Shivnan et al., 1996) in response to extracellular stimuli. Whether these observations relate to the possible sequestration of CD45 by glucosidase II remains speculative and demands further investigation.

F. Structural Consequences of the Interaction between CD45 and Glucosidase II: The "Cover Up" Hypothesis

Although CD45 glucosylation has not been studied, striking differences in the glycosylation of CD45 in mature versus immature-type T cells have been documented. The serum mannan-binding protein, a C-type lectin, has been found to be capable of recognizing CD45 on immature thymocytes ($CD4^+CD8^+CD3^{low}$) but not mature thymocytes, splenocytes, or lymph node cells (Uemura et al., 1996). This lectin recognizes Man (in addition to GlcNAc) and binds to CD45 in an Endo H-sensitive manner, consistent with the observation that immature thymocytes display surface-expression of CD45 glycoforms bearing high-mannose and/or hybrid-type oligosaccharides (Uemura et al., 1996). Intriguingly, these maturation state-dependent differences in glycosylation do not affect thymocyte glycoproteins in a global manner, in that the binding of this lectin to proteins other than CD45 could not be detected. These results provide strong evidence for cell type-specific regulatory influences that selectively impinge on CD45 glycosylation.

The ability of thymic-expressed CD45 to maintain Endo H-sensitive glycans suggests that subsets of CD45 oligosaccharides are excluded from one or more early carbohydrate processing events in these immature cells. ER and Golgi processing pathways leading to the acquisition of Endo H resistance have been well characterized (Fig. 8-2). Successive hydrolysis of α1,2-linked Man residues occurs in the ER and *cis* Golgi, resulting in the generation of a Man_5GlcNA_2 product that is acted upon by GlcNAc Transferase I in the *medial* Golgi. The final step in the acquisition of Endo H resistance occurs when the product of this reaction, $GlcNAcMan_5GlcNAc_2$ is recognized

by Mannosidase II, generating $GlcNAc_1Man_3GlcNAc_2$, which matures to a complex-type *N*-glycan (Tai et al., 1977). Blockade of one or more of these processing steps results in the formation of either high mannose or hybrid-type oligosaccharides, depending on the particular step(s) omitted (Elbein, 1991).

It is tempting to speculate that the ability of glucosidase II to stably associate with CD45 in unfractionated thymocytes (the majority of which are of immature phenotype) but not splenocytes functions as a control mechanism governing the carbohydrate content of CD45. Since steric accessibility is an important determinant in the regulation of carbohydrate processing events (Hsieh et al., 1983), its is possible that the stable binding of glucosidase II to CD45 restricts the formation of complex-type oligosaccharides by blanketing certain carbohydrate structures and thereby inhibiting specific processing steps (Fig. 8-3). According to this "cover up" hypothesis, CD45 may exit the ER with glucosidase II remaining bound. The notion that glucosidase II may traffic beyond the ER is not unreasonable given its altered localization in some cells, and is consistent with the observation that GII_α can be detected at the surface of SAKR cells. Since glucosidase II is capable of partially protecting CD45 oligosaccharides from cleavage by an endoglycosidase recognizing immature *N*-glycans *in vitro*, by extension it may also shield a subset of CD45 oligosaccharides from recognition by ER and/or Golgi mannosidases. Of particular significance is the well-documented ability of glucosidase II to bind (and therefore possibly protect) mannose branches adjacent to the extension bearing α1,3-linked Glc (Grinna and Robbins, 1980). It is possible that the interaction of glucosidase II with CD45 somehow interferes with the putative interaction between the GII_β HDEL

motif and the retrieval receptor. Alternatively, glucosidase II may remain associated with CD45 only as far as the *cis*-Golgi and then undergo retrieval to the ER.

There are a number of precedents supporting the ability of "resident" ER proteins to function as "escorts" for cell surface glycoproteins, a phenomenon that may be unique to immature-type lymphocytes. In pro B cells, for instance, the immunoglobulin α/β heterodimer traffics to the plasma membrane in association with calnexin rather than the B-cell Ag receptor (which is not expressed at this early developmental stage), where it functions to initiate signals associated with cell differentiation (Nagata et al., 1997). In immature thymocytes, calnexin has been found to form protein-protein interactions with a subpopulation of CD3ε molecules transiting to the cell surface in the absence of TCR expression (Wiest et al., 1995), an interaction that results in the retention of Glc-containing Endo H-sensitive linkages (Wiest et al., 1997). This result is doubly significant in that it not only demonstrates that an ER chaperone can confer protection from Golgi processing enzymes, but counteracts dogmatic notions that cell-surface proteins are incapable of retaining Glc linkages. Also provocative is the finding that TCRβ is capable of being expressed in immature but not mature T cells in the absence of TCRα, apparently by virtue of its ability to form disulfide linkages with an 80-kDa acidic protein and a larger protein of undetermined mass, both of which label poorly by surface iodination and are thought to represent ER proteins (Kishi et al., 1991). In fact, in immature thymocytes and BW thymic lymphoma cells numerous subgroups of "resident" ER proteins have been detected at the cell surface (Wiest et al., 1997).

A number of experimental tests can be applied to investigate the "cover up" hypothesis in the context of CD45. Subcellular fractionation studies can be undertaken to

define the intracellular compartments in which CD45 and glucosidase II are associated. Pulse chase analysis, in combination with surface biotinylation, can be used to compare rates of trafficking of glucosidase II and CD45, while treatment with Endo H can be used to assess oligosaccharide maturation. Characterization of the glycosylation status of CD45-associated GII_{α} will be important in clarifying whether glucosidase II transits to the *medial*-Golgi, although the "cover up" model would not preclude a scenario wherein glucosidase II associates with CD45 until the *cis*-Golgi before itself recycling to the ER. To further assist in dissecting the trafficking characteristics of these proteins, intracellular transport pathways can be disrupted by temperature shift or with drugs such as monensin or brefeldin A. Immunohistochemical studies may be informative in identifying the subcellular compartments in which glucosidase II and CD45 colocalize. It will be particularly interesting to determine whether these variables differ in mature and immature T cells. In addition, CD45-deficient cells can be exploited to elucidate effects of CD45 expression on localization and trafficking of glucosidase II, while the $PHA^{R}2.7$ mutant can be utilized to address the converse issue.

G. Biological Relevance of CD45 Glycosylation

The phenomenon of *N*-linked glycosylation, in general, is considered to be important in regulating protein stability, folding, and intracellular targeting (Helenius, 1994). Studies employing the inhibitor tunicamycin have revealed that *N*-glycosylation of CD45 is essential for its transport beyond the Golgi apparatus (Pulido and Sanchez-Madrid, 1992). Carbohydrate determinants are also thought to be crucial for certain

recognition processes at the cell surface, such as cell-cell interactions occurring during embryogenesis (Helenius, 1994). Variation in the *N*-glycan composition of CD44 has been found to influence its ability to interact with its cognate ligand, hyaluronan (Lesley et al., 1995). In the case of CD45, the B-cell adhesion molecule CD22 has been shown to recognize $CD45^{Null}$ glycoforms expressed on T cells (Stamenkovic et al., 1991). Because recognition is restricted to an α2,6-linked sialic acid modification that is present on a variety of proteins (including CD75), CD22 is not strictly considered a specific ligand for CD45, although this semantic consideration does not preclude the possibility that this interaction has significant biological consequences. It does, however, complicate the interpretation of experiments demonstrating that the interaction of CD22 with CD45 (and possibly other sialyated surface proteins) enhances tyrosine phosphorylation and second messenger production in response to sub-optimal stimulation with anti-CD3 mAb (Sgroi et al., 1995). In addition to CD22, a secreted S-type cation-independent lectin known as galectin-1 interacts with an oligosaccharide moiety on CD45 glycoforms preferentially expressed by immature thymocytes (Baum et al., 1995). As is the case with CD22, the structure recognized by galectin-1, β-galactoside, is present on *N*-glycans of other proteins such as CD43. Nevertheless, a subset of activated T cells are induced to undergo apoptosis within 30 minutes of galectin-1 treatment, an effect than can be blocked by CD45 mAb (Perillo et al., 1995). Intriguingly, recent data indicate that IL-2 is a bifunctional ligand possessing a receptor-binding domain and a distinct lectin domain capable of recognizing high-mannose structures (Zanetta et al., 1996). Occupation of both ligand-binding domains may allow the IL-2 receptor, the β-chain of which associates with $p56^{lck}$ (Perlmutter et al., 1993), to become juxtaposed with CD45

glycoforms bearing these oligosaccharides. Cumulatively, these data indicate that a variety of soluble or membrane-associated proteins may be capable of recognizing select CD45 glycoforms on the same or apposing cell surface, in turn eliciting distinct biological outcomes.

A particularly confounding aspect of CD45 biology that has remained unresolved is why multiple isoforms of the protein exist and are regulated according to cell type, developmental stage, and differentiation state. The ability of CD45 isoforms to serve as lineage-specific markers has led to the hypothesis that specific ligands must exist for these isoforms; however, these have eluded identification. One reason why previous approaches may have failed in this endeavor is that cloning and expression methodologies employing non-mammalian cells and/or non-hematopoietic lineages necessarily compromise the authenticity of CD45 glycosylation. One explanation for the dramatic differences in the oligosaccharide composition of CD45 between immature and mature T cells is that carbohydrate determinants function to modulate ligand interactions. If, for example, CD45 retains α1,3-linked Glc due to lack of cleavage by glucosidase II in immature-type cells, this moiety may form a recognition structure. In addition to the ability of oligosaccharide determinants to directly mediate ligand binding, they may also influence the conformation of CD45 and/or its membrane diffusion characteristics, properties that may indirectly impinge on the ability of CD45 to interact with other extracellular, transmembrane, or cytoplasmic proteins. Interestingly, evidence has been obtained from Ab co-capping experiments that different isoforms of CD45 selectively associate with distinct cell surface proteins (Dianzani et al., 1992; Leitenberg et al., 1996). Furthermore, distinct CD45 isoforms, when individually expressed in CD45-

deficient T cells, differentially influence activation outcomes in the context of APC, but not Ab, stimulation (Novak et al., 1994; Leitenberg et al., 1996). Based on these considerations, it may be theorized that the primary biological rationale for the generation of distinct CD45 glycoforms (i.e. differentially glycosylated isoforms) is to regulate the interaction of CD45 with other proteins.

H. Cellular Outcomes of CD45 Ligation

In the absence of information pertaining to the identity of specific CD45 ligands, a variety of experimental models have been invoked to study potential biological outcomes of CD45 ligation. The results yielded by these studies have been as diverse as the methodologies employed and thus raise the possibility that CD45 ligands may exert pleiotropic effects and/or that the effects of CD45 ligation may be different in different cell types. A number of groups have examined the effects CD45 crosslinking in the context of Ag receptor triggering. Soluble CD45 mAbs modulate lymphocyte activation-specific pathways both positively (Schraven et al., 1989; Wolff and Janeway, 1994) and negatively (Goldman et al., 1992; Kawauchi et al., 1994; Maroun and Julius, 1994a; Shivnan et al., 1996). Ab engagement of CD45 on immature $CD4^+CD8^+$ thymocytes augments $p56^{lck}$ activity and inhibits cell differentiation (Benveniste et al., 1994). Crosslinking of CD45 also enhances the respiratory burst in phagocytes, indicating that the capacity of CD45 ligation to regulate functional responses is not limited to T and B lymphocytes (Liles et al., 1995). Dimerization of the CD45 cytoplasmic domain by an artificial ligand inhibits proximal TCR-induced signals (Desai et al., 1993), an effect that

may be due to catalytic site occlusion by an inhibitory wedge structure induced during dimer formation (Majeti et al., 1998). Although biochemical evidence for dimerization of cell-surface CD45 has not been obtained, the catalytic domain of a related PTP, Receptor PTPα, has been found to crystallize in an inhibitory dimer-type structure (Bilwes et al., 1996). In the context of lymphocyte activation, ligand-induced downregulation of the PTP activity of CD45 may occur subsequent to PTK activation, allowing for the accumulation of phosphotyrosine in the microenvironment of the contact cap.

Several studies have modeled the outcomes of CD45 ligation in the absence of Ag-receptor stimulation. Treatment of cells with soluble CD45 mAb induces homotypic adhesion that is frequently (Lorenz et al., 1993; Bernard et al., 1994; Spertini et al., 1994), but not exclusively (Zapata et al., 1995), mediated by the LFA-1 integrin. Conversely, CD45 mAbs have also been observed to impair integrin-mediated adhesion events (Arroyo et al., 1994; Hanaoka et al., 1995). Recent data indicate that the regulation of integrin function by CD45 is mediated by Src family PTKs (Roach et al., 1997). Immobilized CD45 mAbs trigger rapid cytoskeletal changes in mouse T cells in conjunction with PTK activation and phosphorylation of $p56^{lck}$-associated proteins (Arendt et al., 1995), a phenomenon that may also relate to regulation of adhesive events. Interestingly, prolonged (~6-8 h) engagement of CD45 on an immobilized surface has been found to induce apoptosis in Jurkat cells, an effect enhanced by co-immobilization of anti-CD3 mAb and inhibited by herb A (Klaus et al., 1996).

The ability of CD45 ligation to trigger Ag receptor-independent outcomes is significant in the context of potential novel functions of CD45 in the wide variety of cell

types in which it is normally expressed. The capacity of CD45 to couple to cytoskeletal reorganization and cell adhesion pathways raises the possibility that protein-protein interactions involving the CD45 ectodomain may influence cellular migration events. The CD45 extracellular segment is thought to play a central role in regulating adhesive events central to thymic maturation (Pilarski, 1993), and thymic maturation is severely undermined in CD45-deficient mice (Byth et al., 1996). Alternatively, the ability of CD45 ligation to trigger a subset of TCR-triggered phosphorylation events (Arendt et al., 1995) may allow CD45 ligands to augment responses, allowing CD45 to function in a costimulatory manner to reduce activation thresholds. This view is also consistent with the ability of CD45 mAbs to trigger apoptosis, since prolonged stimulation through the TCR leads with even more rapid kinetics to programmed cell death (Glickstein et al., 1996; Klaus et al., 1996). Interestingly, a recent study revealed that ligation of CD45 in NK cells is capable of upregulating $p56^{lck}$ activity associated with enhanced cytokine production (Xu and Chong, 1995), suggesting that CD45 may act in a costimulatory capacity in this cell type also. If CD45 is capable of being engaged *in vivo* in such a manner that costimulatory signals are elicited, this would call into question the model of Shaw and Dustin (1996) stipulating that CD45 must be physically excluded from the contact cap to allow for accumulation of phosphotyrosine. The view that CD45 engagement generates stimulatory signals also conflicts with the results of an influential study employing a CD45/EGFR chimera in which downregulation of cellular tyrosine phosphorylation was observed upon ligand addition (Desai et al., 1993). Unfortunately, it has not been possible to assess the PTP activity of CD45 while in a clustered or dimeric configuration. Thus, it is not possible to exclude the possibility that these experimental

manipulations act solely to influence the protein-protein interactions in which CD45 engages, rather than modulating its enzymatic activity. Given that CD45 is capable of regulating Src family PTKs in a dichotomous fashion that is subject to cell type-specific controls (Ostergaard et al., 1989; Ostergaard and Trowbridge, 1990; Cahir McFarland et al., 1993; Hurley et al., 1993; Biffen et al., 1994; Burns et al., 1994; Roach et al., 1997; Stone et al., 1997), it is difficult to predict, based on PTK activity alone, whether the PTP activity of CD45 is upregulated or downregulated in these systems.

The diverse and conflicting results of experimental systems designed to explore outcomes associated with artificially induced CD45 ligation must serve as a caution against making hasty conclusions regarding the mechanisms by which signals transmitted through the CD45 ectodomain may modulate its function. A challenging but important future goal must be to identify and characterize proteins that interact with the ectodomains of authentically glycosylated CD45 isoforms derived from a variety of cell types. Only then will it be possible to definitively assess whether the extracellular domains of distinct CD45 glycoforms act in novel ways in different subsets of cells. Experiments aimed at reconciling the structural complexity of the extracellular domain with the intracytoplasmic functions of CD45 are likely to provide new insights into the complex workings of the mammalian immune system and, more specifically, the mechanisms by which *N*-linked glycosylation may impinge on recognition processes.

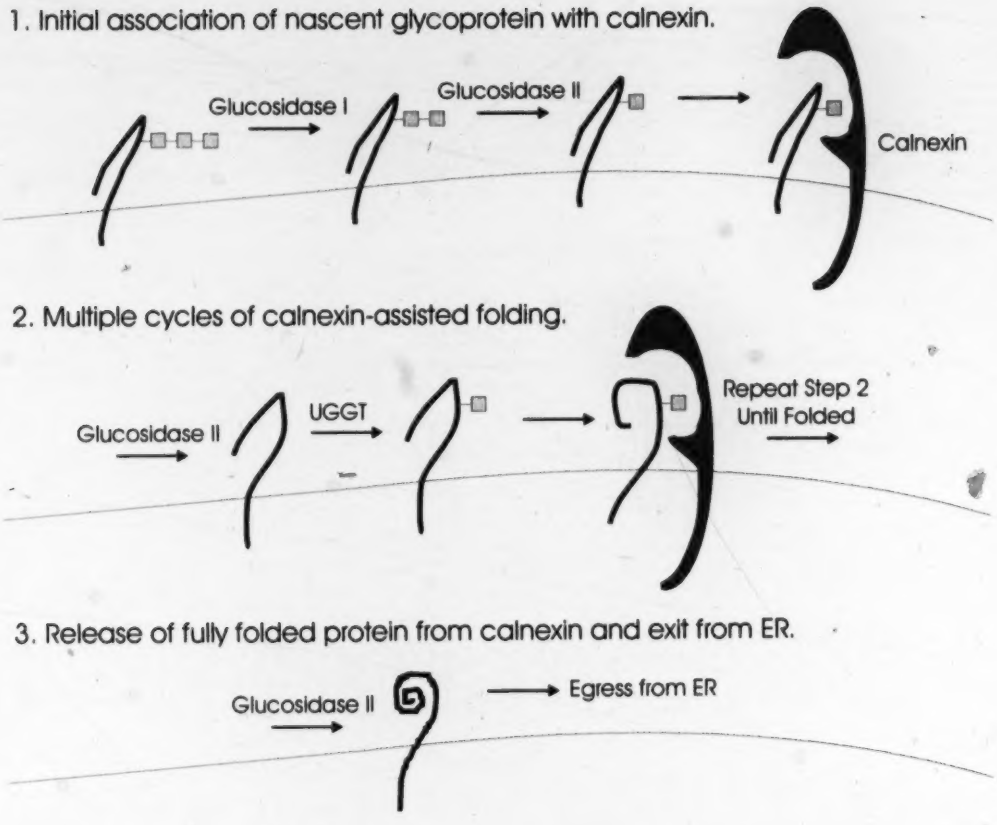

FIGURE 8-1. Coordination of carbohydrate processing and protein folding in the endoplasmic reticulum. The flow diagram depicts three stages of glycoprotein maturation in the ER. In the first stage, trimming of the outermost 2 Glc residues from immature *N*-glycans by glucosidases I and II generates a monoglucosylated substrate for the lectin domain of calnexin (and/or calreticulin, not shown). The association with calnexin allows the substrate protein to undergo one cycle of folding. In the second phase of the maturation pathway, glucosidase II acts on the partially folded protein to induce transient dissociation from calnexin. The UDP-Glc:glycoprotein glucosyltransferase (*UGGT*) then re-glucosylates the substrate protein, allowing it to re-associate with calnexin. This cycle of de- and re-glucosylation is repeated until the substrate glycoprotein achieves native conformation. In the final stage of maturation, the glycoprotein is acted upon by glucosidase II but not the transferase, permitting its permanent release from calnexin and subsequent egress from the ER.

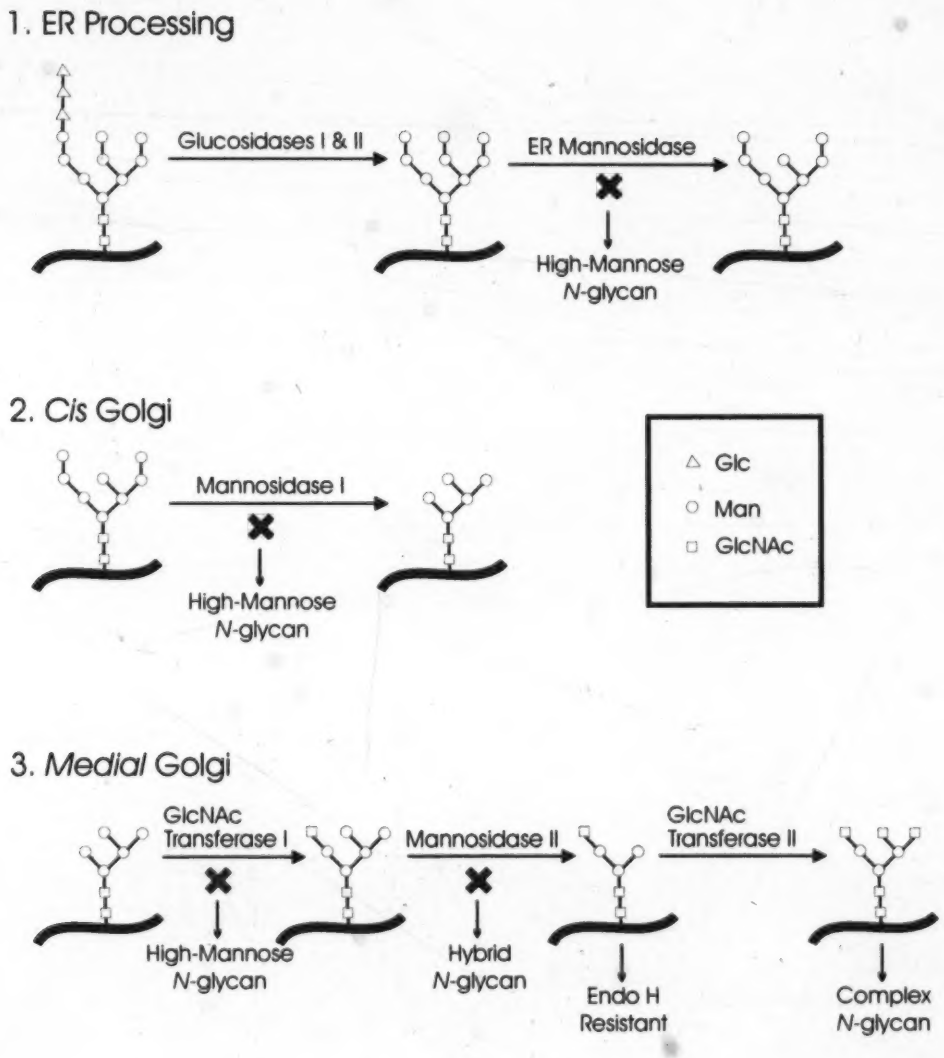

FIGURE 8-2. Overview of carbohydrate processing reactions leading to acquisition of Endo H resistance. Shown are the processing steps involved in the biogenesis of complex *N*-glycans. Blockade of these steps may result in the generation of high-mannose or hybrid-type oligosaccharide species. Note that processing by mannosidase II in the *medial* Golgi is required for the transition to Endo H resistance, a reaction step dependent on the completion of previous processing events and associated with the transition to complex-type glycans. (Adapted from Elbein, 1991).

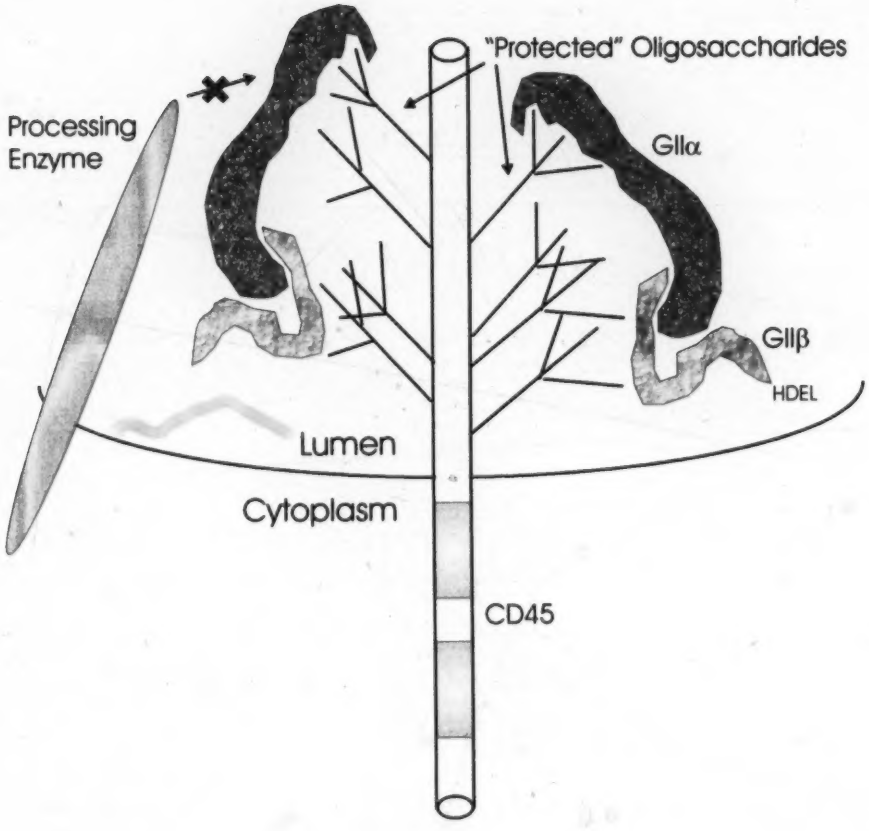

FIGURE 8-3. "Cover up" model. According to the "cover up" hypothesis, in certain cells the association of glucosidase II with CD45 is sufficiently stable and long-lived to provide protection from ER and/or Golgi processing enzymes. Consequently, subsets of *N*-glycans on CD45 fail to mature to complex-type oligosaccharides.

CHAPTER IX

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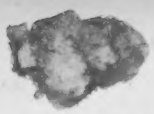

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EDUCATION

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1988-1993	Faculty of Biological Sciences, University of Alberta B.Sc. Genetics (First Class Honors)

ACADEMIC HONORS

- Fellowship Award, Medical Research Council of Canada (1998-)
- Studentship Award, Alberta Heritage Foundation for Medical Research (1993-1998)
- Studentship Award, Natural Sciences and Engineering Research Council of Canada (1993-1997)
- Walter H Johns Graduate Scholarship, University of Alberta (1993-1997)
- Marie Arnold Cancer Research Graduate Scholarship (1997)
- Student Travel Award, National Cancer Institute of Canada (1997)
- Second Place Poster Award, Canadian Society for Immunology Spring Meeting (1997)
- Mary Louise Imrie Graduate Student Travel Award (1996)
- Travel Award, Canadian Society for Immunology (1994)
- Best Oral Presentation, Faculty of Medicine Student Research Day (1992)
- University of Alberta Max Wyman Entrance Scholarship (1988)

PUBLICATIONS

1. Arendt, C.W., G. Hsi and H.L. Ostergaard. (1995) Immobilized antibodies to CD45 induce rapid morphologic changes and increased tyrosine phosphorylation of p56lck-associated proteins in T cells. *J Immunol* 155: 5095.

2. Arendt, C.W. and H.L. Ostergaard. (1995) CD45 protein-tyrosine phosphatase is specifically associated with a 116-kDa tyrosine-phosphorylated glycoprotein. *J Biol Chem* 270: 2313.

3. Arendt, C.W. and H.L. Ostergaard. (1997) Identification of the CD45-associated 116-kDa and 80-kDa proteins as the α- and β-subunits of α-glucosidase II. *J Biol Chem* 272: 13117.

4. Ostergaard, H.L., O. Lou, C.W. Arendt, and N.N. Berg. (1998) Paxillin phosphorylation and association with Lck and Pyk2 in anti-CD3- or anti-CD45-stimulated T cells. *J Biol Chem* 273: 5692.

ABSTRACTS AND PRESENTATIONS

1. Arendt, C.W. and H.L. Ostergaard. Identification of p116, a CD45 tyrosine phosphatase-associated protein. *The 8th Spring Meeting of the Canadian Society for Immunology*, St. Adele, 1994 (poster).

2. Arendt, C.W. and H.L. Ostergaard. Antibodies to CD45 induce rapid morphological changes in T cells. *The 9th International Congress of Immunology*, San Francisco, 1995 (oral and poster presentations).

3. Arendt, C.W. and H.L. Ostergaard. Purification and cDNA cloning of mouse glucosidase II by virtue of its interaction with CD45 glycoprotein. *The 11th Spring Meeting of the Canadian Society for Immunology*, Lake Louise, 1997 (poster).

4. Arendt, C.W. and H.L. Ostergaard. CD45 transmembrane phosphatase physically associates with the mouse homologue of human 80K-H. *Symposium on Temporal and Spatial Determinants of Specificity in Signal Transduction*, Keystone, 1997 (poster).

5. Arendt, C.W. and H.L. Ostergaard. The interaction between the α- and β- subunits of glucosidase II is mediated by the amino terminus of the β-subunit protein. *The 17th International Congress of Biochemistry and Molecular Biology*, San Francisco, 1997 (poster).

PERSONAL DEVELOPMENT

- Student representative on Immunology Network Executive Committee, which oversees a bi-monthly seminar series that includes student-sponsored speakers.
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- Teaching assistant for undergraduate Immunology course.
- Supervision of summer students.
- Volunteer judge at annual city-wide Science Fair.
- Volunteer, Metro Cinema Film Society.
- Enjoy reading, photography, swimming, music, cinema.